Temperature dependence of lysozyme hydration and the role of elastic energy

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Water plays a critical role in protein dynamics and functions. However, the most basic property of hydration—the water sorption isotherm—remains inadequately understood. Surface adsorption is the commonly adopted picture of hydration. Since it does not account for changes in the conformational entropy of proteins, it is difficult to explain why protein dynamics and activity change upon hydration. The solution picture of hydration provides an alternative approach to describe the thermodynamics of hydration. Here, the flexibility of proteins could influence the hydration level through the change of elastic energy upon hydration. Using nuclear magnetic resonance to measure the isotherms of lysozyme *in situ* between 18 and 2 °C, the present work provides evidence that the part of water uptake associated with the onset of protein function is significantly reduced below 8 °C. Quantitative analysis shows that such reduction is directly related to the reduction of protein flexibility and enhanced cost in elastic energy upon hydration at lower temperature. The elastic property derived from the water isotherm agrees with direct mechanical measurements, providing independent support for the solution model. This result also implies that water adsorption at charged and polar groups occurring at low vapor pressure, which is known for softening the protein, is crucial for the later stage of water uptake, leading to the activation of protein dynamics. The present work sheds light on the mutual influence of protein flexibility and hydration, providing the basis for understanding the role of hydration on protein dynamics.

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I. INTRODUCTION

Hydration plays an important role in protein folding, dynamics, and functions [1–4]. For instance, the enzymatic activity of lysozyme increases significantly above a hydration level of $h = 0.2$ (in grams of water per gram of dry proteins) [2,5]. Water-protein interaction has also been a central theme of protein folding ever since Kauzmann introduced the concept of hydrophobic interaction in 1959 [1]. One of the direct ways to probe the characteristics of protein hydration is by water sorption isotherms. Surprisingly, the mechanism governing water sorption isotherms in proteins still remains an issue of debate [2,6]. The prevailing picture of surface adsorption does not take into account structural and dynamical changes of proteins associated with hydration. The present work provides experimental evidence indicating that the understanding of water sorption isotherms in the globular protein lysozyme also requires a treatment beyond surface adsorption theory. A deeper understanding of water sorption isotherms in proteins could shed light on the structure, dynamics, and protein functions [2–10].

The characteristics of water sorption isotherms of globular protein powders are quite universal near room temperature [2,6,11,12]. From a dry condition to a relative water vapor pressure $P/P_0 \sim 0.7$ (P_0 is the saturated water vapor pressure at a given temperature), the amount of absorbed water increases gradually with P/P_0, reaching a certain hydration level ($h \sim 0.2$ in lysozyme). This initial stage of hydration is identified with water adsorption at ionizable groups, and charged and polar sites, as illustrated in Fig. 1(a) [2,11,13,14]. Above this pressure, an upswing in water uptake occurs. It has been suggested that this upswing in water uptake correlates with the onset of key protein functions [2,13]. So far there is no consensus with regard to the mechanism of this upswing in water uptake [2,6]. The understanding of the nature of this upswing in water uptake is the focus of this work. In particular, the temperature dependence of this upswing is clearly established at temperatures below 8 °C. Although hydration near the physiological temperature is of primary concern in biology, the temperature dependence of the isotherm below room temperature provides important clues on the mechanisms of hydration. To the best of our knowledge, water sorption isotherms over the entire pressure range of $P/P_0 = 0$ to 1 have not been reported for protein crystallites down to temperatures near 0 °C. This could be due to the inconvenience of using the traditional gravimetric technique for *in situ* hydration measurements over a wide range of vapor pressure and temperature. In this work, water sorption isotherms were measured with an alternative approach based on ^{1}H nuclear magnetic resonance (NMR) [15,16].

II. EXPERIMENTS

The hen egg-white lysozyme (HEWL) (catalog no. L-7561, 3× crystallized, dialyzed, and lyophilized) was purchased from Sigma Aldrich and used without further purification. The enzymatic function of lysozyme is strongly affected by the hydration level and the temperature: It increases significantly above a hydration level of $h = 0.2$ [5]; the activity at 10 °C is only ∼20% of that at 35 °C [17]. The lysozyme is loaded into the quartz NMR sample tube connected to an *in situ* water loading system with controlled vapor pressure and

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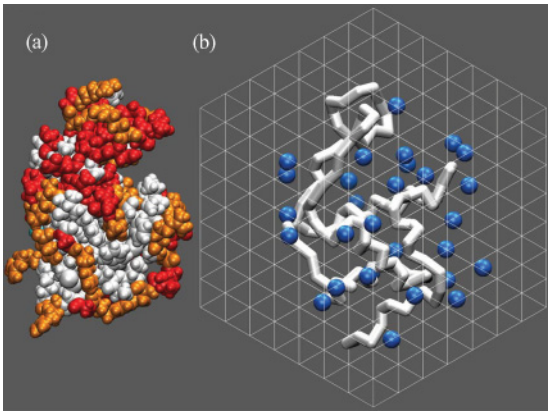

FIG. 1. (Color online) (a) A van der Waals representation of a lysozyme molecule colored with a hydrophobic scale. White: hydrophobic; red (dark gray): hydrophilic; orange (gray): positive or negative charge. (b) A lattice model of protein hydration. The white chain represents a lysozyme molecule occupying x lattice cells. The blue (gray) spheres are hydration water, each of which occupy one lattice cell. Their locations are obtained from hydration water of <0.34 nm away from the protein in molecular dynamics simulations shown in Sec. III F.

temperature [16]. A single pulse (~ 5 μs) was used to excite the ^{1}H NMR signals at 0.8 T (34 MHz ^{1}H NMR frequency). The spectrum of as-received lysozyme powder contains two major components: a sharp peak with the full width at half maximum (FWHM) of $\sim$2.4 kHz on top of a broad peak with FWHM of $\sim$40 kHz, as shown in Fig. 2(a). The sharp peak can be gradually removed by pumping the sample chamber to 10^{-3} Torr for 24 h at room temperature. The broad peak remains unchanged during the pumping procedure and was assigned to proton background solely from lysozyme molecules [12]. After the initial pumping, a constant water vapor pressure was kept in the sample chamber at each step to rehydrate the lysozyme. The intensity of the NMR signal reaches equilibrium within 2–3 h, much faster than the isopiestic method that is slowed down by the air environment [18]. No further change in NMR signal was observed after 3 h as verified by continued monitoring over a time period of 2 days. The sharp peak reappears in the rehydration process and is assigned to water sorption in the protein [12]. Since the protons account for $\sim$6.8% weight of a lysozyme molecule [19], the hydration level h can be calculated from the intensity ratio of the sharp and broad peaks of NMR spectra at equilibrium. Because the NMR signal is very stable and accurate, the major systematic error comes from the conversion from the intensity ratio of broad and narrow peaks into the hydration level. The effect of the detection delay (~ 8 μs) on the fast decay component of the free-induction decay, corresponding to the broad peak in spectra, has to be considered. Based on a Gaussian decay, we can extrapolate it back to the end of the excitation pulse [20]. The extrapolation leads to a systematic error of $\sim$5% to the conversion coefficient. The similarity of sorption isotherm at 18 °C measured by NMR and other methods confirms that this error is not significant [21]. Such an error only affects the absolute hydration level. Its net effect is similar to stretching or shrinking the hydration axis of isotherms by $\sim$5%. It has no effect on the relative value for measurements at different temperatures and at different vapor pressures. Thus the shape of sorption isotherms remains unaffected and the systematic error on the absolute value of the hydration level is insignificant. The isotherms measured by ^{1}H NMR method were shown to be consistent with those obtained by the traditional gravimetric technique [12].

III. RESULTS AND DISCUSSION

Figure 3 shows measured hydration level h versus relative vapor pressure P/P_0 in lysozyme at temperatures of 18, 8, 4, and 2 °C. These isotherms share a sigmoidal feature in common with the type II isotherm [22]. The isotherms at low relative pressure ($P/P_0 < 0.7$) depend linearly on P/P_0 with small changes in slope below 8 °C. The adsorbed water in this part of the isotherm has been correlated with the charged and polar groups in proteins based on *in situ* IR measurements [11,13]. Above $P/P_0 = 0.7$, the measured isotherms depend strongly on temperatures over a small range of temperatures, such as from 18 to 2 °C. This dependence has not been established by earlier studies [2,6].

There is a seemingly dramatic change between isotherms at 8 and 4 °C. This is caused by the difference in water uptake

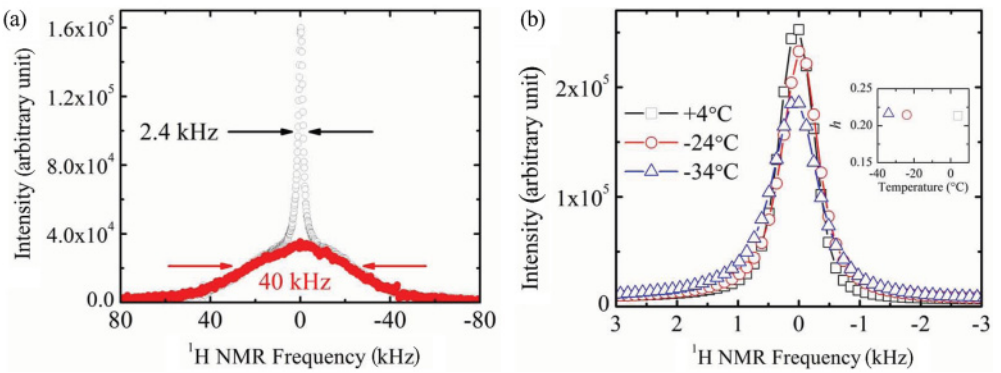

FIG. 2. (Color online) (a) NMR spectra of lysozyme powder before (open circle) and after pumping (filled circle). (b) The sharp NMR peak corresponding to the hydration water of protein as temperature decreasing from 4 to –34 °C. The initial hydration level is $h \sim 0.21$ at 4 °C. The inset shows the hydration levels estimated from the integrated intensities of such sharp peaks.

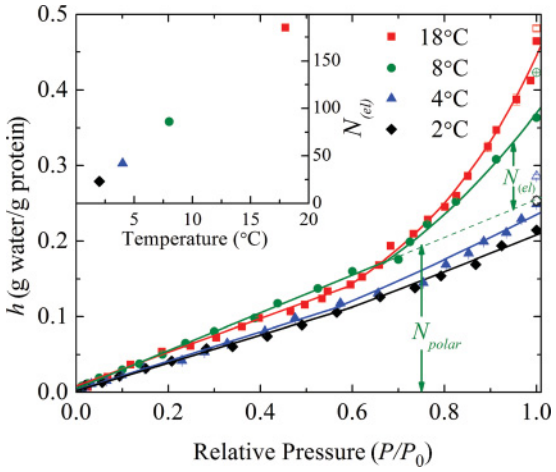

FIG. 3. (Color online) Water sorption isotherms in lysozyme powder from 18 to 2 °C. The isotherms at high relative pressure are fitted as the implicit form of Eq. (2) with parameters shown in Table I. Inset: $N^0_{(el)}$ versus temperature.

on the charged and polar groups at low relative pressure. The portion of hydration water related to the upswing actually decreases smoothly from 18 and 2 °C, when the number of water molecules adsorbed on polar groups is removed. We will discuss this in Sec. III E. As pressure reaches saturated vapor pressure, further hydration will cause condensation of bulk water without any increase of pressure. The upper data points at $P/P_0 = 1$, as shown in open symbols, correspond to such bulk condensation, confirming that we reached saturated vapor pressure at corresponding temperatures.

A. Surface adsorption picture

The main reason for the lack of isotherm data for low temperatures near 0 °C is perhaps the prevailing belief that the isotherms should be largely temperature independent when plotted versus P/P_0 [6]. In the picture of surface adsorption the upswing in water uptake above $P/P_0 \sim 0.7$ is attributed to multilayer formation [6,11,23]. The best known surface adsorption theory for describing multilayer formation is the Brunauer-Emmett-Teller (BET) theory [22]. The BET isotherm is given by

$$h \propto \frac{(c-1)\,P/P_0}{1+(c-1)\,P/P_0} + \frac{P/P_0}{(1-P/P_0)}, \qquad (1)$$

where $c = \tau/\tau_1$. τ and τ_1 are the residence times of molecules directly bound to the adsorbent surface and adsorbed on top of the first layer, respectively. Inside protein crystals, spatial restrictions would prevent bulk water condensation and adsorption is limited to a finite number of layers and can be easily taken into account in the BET theory [22,23]. Other surface adsorption models give somewhat different expressions for the isotherms, but the essence for the description of the upswing in water uptake is very analogous to the standard BET theory [6,23]. Equation (1) shows that the upswing in water uptake, as dominated by the second term, is expected to be temperature independent when plotted versus the relative pressure P/P_0 [6]. Isotherm data taken above room temperature indeed confirm this expectation [21,24]. However, this is no longer the case in lysozyme below 10 °C. The upswing in water uptake above $P/P_0 \sim 0.7$ gradually disappears as the temperature approaches 0 °C. Such temperature dependence of water sorption isotherms cannot be explained in the framework of surface adsorption [22,23].

B. Modified Flory-Huggins theory

It was suggested that the system at the upswing could be treated as a very concentrated solution based on the Flory-Huggins theory [6,25]. The Flory-Huggins theory describes the mixing of water with protein by a three-dimensional lattice model, where a protein molecule is represented by a chain occupying x lattice cells and each water molecule occupies one lattice cell [26]. This model is illustrated in Fig. 1(b). Upon mixing of N_1 water molecules with N_2 protein molecules, the change of the free energy is given by $\Delta G = \Delta H_m - T\Delta S_m + \Delta H_{(el)}$, where $\Delta H_m = k_B T \chi N_1 N_2/(N_1 + xN_2)$ is the enthalpy of mixing characterized by the parameter of interaction χ between water and the protein molecule and k_B is the Boltzmann constant. $\Delta S_m = -k_B\,[N_1 \ln v_1 + N_2 \ln v_2]$ is the entropy of mixing where $v_1 = N_1/(N_1 + xN_2)$ and $v_2 = xN_2/(N_1 + xN_2)$ are the volume fractions of water and proteins, respectively. $\Delta H_{(el)} = V\,(K/2)\,(v_2^{-1/3} - 1)^2$ is the elastic energy, as approximated to the first order, due to the volume expansion upon water addition as given by Rowen and Simha [25], where $V = \overline{V_1}\,(N_1 + xN_2)$ is the total volume of the N_2 protein molecules mixed with N_1 water molecules and $\overline{V_1}$ is the volume of each lattice cell. K is the macroscopic parameter of the elastic modulus of the proteins. Since globular proteins have residues that are compact, the core may not be readily available for mixing with water molecules [27]. This can be taken into account by replacing x with ηx in the expressions of ΔH_m and ΔS_m, where $0 \leqslant \eta \leqslant 1$ is the fraction of flexible segments in the protein [28,29]. This correction is not necessary in $\Delta H_{(el)}$ because the elastic energy depends on the volume change of the bulk. The equality of the chemical potentials of water in the vapor phase, $\mu_{vapor} = k_B T \ln(P/P_0)$, and in the water-protein mixture leads to [25,28,29]

$$[\ln(P/P_0) - \eta \ln v_1 - \eta v_2]\, v_2^{-2} = \chi + \frac{K\overline{V}_1}{2k_B T}\left(\frac{1}{v_2^{1/3}} - 1\right)\left(\frac{5}{3v_2^{1/3}} - 1\right) v_2^{-2}. \qquad (2)$$

Here, the plot of $[\ln(P/P_0) - \eta \ln v_1 - \eta v_2]v_2^{-2}$ versus $(v_2^{-1/3} - 1)\,[(5/3)v_2^{-1/3} - 1]v_2^{-2}$ is linear with the intercept at χ and the slope containing the elastic modulus K, if both parameters are constant within a certain range of relative pressure. Using the partial specific density of water ($\rho_1 = 1.0$ g/cm^3) and lysozyme ($\rho_2 = 1.38$ g/cm^3) [30], v_1 and v_2 can be determined from the hydration level h via $v_1 = 1 - v_2$ and $v_2 = 1/(h\rho_2/\rho_1 + 1)$. The exact value of η is unknown. We could examine a wide range of η values and obtain the corresponding elastic modulus K.

As pointed out already by Rowen and Simha, $\Delta H_{(el)}$ was estimated to be too small for polymers and proteins at room temperature to have any significant effect on sorption isotherms [25]. This is consistent with the largely temperature-independent water sorption isotherms above room temperature

[6]. The key idea of this work is that this situation might change at lower temperatures because K increases strongly with decreasing temperature. For instance, the Young's modulus of lysozyme increases more than fivefold as temperature decreases from 53 to 22 °C based on mechanical measurements [31]. The elastic modulus K should be similar to Young's modulus if the Poisson's ratio for lysozyme is assumed to be 0.33 [32]. This suggests that the elastic energy could have significant effect on water sorption isotherms below room temperature. The elastic energy term also provides a unique link between the microscopic mechanism of water sorption and a macroscopic quantity K, which can be compared to values obtained by mechanical measurements. This offers an independent validation of the solution picture of protein hydration.

C. The elastic constant and its temperature dependence

Figure 4 plots the isotherm data following Eq. (2) when $\eta = 1$. All isotherms at temperatures from 18 to 2 °C have a linear portion when $[\ln(P/P_0) - \ln v_1 - v_2]\, v_2^{-2} > 0.6$, which corresponds to $P/P_0 > 0.7$. Using 18 cm^3/mol to estimate the water molecule volume $\overline{V}_1$ in Eq. (2), χ and K can both be determined from the linear fits in Fig. 4. These values are listed in Table I. χ is expected to be a constant at high relative pressure and depends on temperature following $\chi = \beta + \alpha/(RT)$ with α and β being constants and R is the gas constant [25,26]. With the temperature change of 18 °C to 2 °C, the change of χ should be <4% with typical values of α (–1.1 kJ/mol) and β (1.3) as determined in serum albumin [25]. From Table I, χ values at different temperatures are all close to 0.59 within errors. This is comparable to the value of 0.87 for water-serum albumin system and 0.41 for water-salmine system [25]. The determined elastic modulus K increases from 190 MPa at 18 °C to 1326 MPa at 2 °C. Although the temperature is in the vicinity of the freezing point of bulk water at 0 °C, such a dramatic change of the elastic modulus is not due to the crystallization of hydration water. Figure 2(b) shows that as the temperature decreases from 4 to –34 °C, the sharp NMR peak corresponding to the nonfreezing hydration water becomes broader. Their integrated intensities, however, remain the same as shown in the inset of Fig. 2(b). This confirms that the freezing temperature of the protein-water system is reduced well below 0 °C [6,20,33]. It is intriguing to note that the Young's modulus of lysozyme tetragonal crystal along [001] direction determined by mechanical measurements increases from 34 MPa at 53 °C to 175 MPa at 22 °C, as shown in the inset of Fig. 4 [31]. Such elastic moduli are shown to be identical to those of amorphous samples [34]. The values of K derived from the isotherms are consistent with the independent mechanical measurement. This provides strong evidence that water sorption above the upswing in the isotherms should be interpreted by the solution model with explicit consideration of the elastic energy and entropy.

For the small globular protein lysozyme, the flexible residues that involve in the mixing near the surface take up a large portion of the total volume. Thus, it is reasonable to assume that the parameter related to partial flexibility, η, is close to 1. Nevertheless, the elastic modulus K does not depend sensitively on η. As shown in Table I, the extrapolated

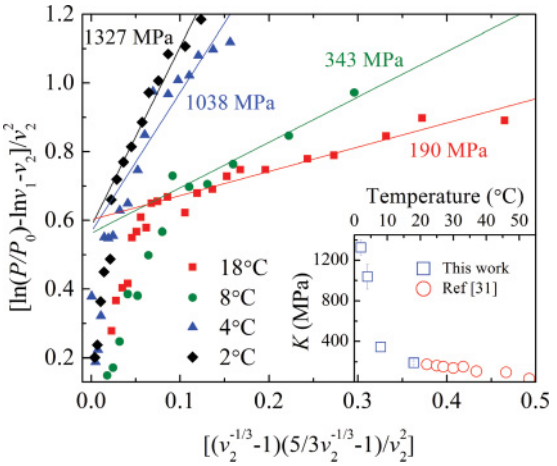

FIG. 4. (Color online) Plots of $[\ln(P/P_0) - \ln v_1 - v_2]v_2^2$ versus $[(v_2^{-1/3} - 1)(5/3v_2^{-1/3} - 1)/v_2^2]$ for the isotherms data following Eq. (2). Plots show linear portion when $[\ln(P/P_0) - \ln v_1 - v_2]v_2^2 > 0.6$. The fitting parameters are shown in Table I for $\eta = 1$. Inset: Comparison of the temperature dependence of the elastic modulus (blue square) derived from isotherms in this study, with the Young's modulus of hydrated lysozyme (red circle) measured in [31].

K remains on the same order of magnitude with $\eta = 1, 0.5$, and 0.25. The temperature dependence of K remains similar for a wide range of η. A change of η does not affect the conclusion that the temperature dependence of the elastic modulus K of the protein is responsible for the observed temperature dependence of the upswing in water uptake. The purpose of this work is not to use sorption isotherms for measuring the elastic modulus quantitatively. Such an agreement on the same order of magnitude is the most we can expect based on the current model. The parameter related to the water-protein interaction, χ, however, depends sensitively on the absolute value of η. The value of χ became uncertain with an unknown η. For this reason, it may be difficult to obtain exact values η for much larger proteins such as bovine serum albumin (BSA) and hemoglobin.

D. Protein properties with hydration above $h = 0.2$

In the above analysis, the elastic modulus K is assumed to be a constant above $P/P_0 = 0.7$. This is supported by a previous measurement which shows that the Young's modulus decreases significantly at low hydration level but depends only

TABLE I. Fitting parameters (χ and K) and standard errors following Eq. (2), when $\eta = 1$, 0.5, and 0.25, and the maximum number of water molecules per protein associated with the elastic free energy $N^0_{(el)}$ and the total number of hydration water molecules per protein $N^0_{hydration}$, at $P/P_0 = 1$.

	χ	K (MPa)	K (MPa)			
T (°C)	$\eta = 1$		$\eta = 0.5$	$\eta = 0.25$	$N^0_{(el)}$	$N^0_{hydration}$
18	0.60±0.01	190±15	353±27	417±35	185	360
8	0.56±0.03	343±45	605±73	709±80	86	299
4	0.57±0.05	1038±137	1628±193	1923±222	42	189
2	0.58±0.03	1327±91	2206±189	2646±241	23	167

weakly on hydration above $h = 0.2$, which corresponds to $P/P_0 0.7$ [34]. This is also consistent with studies of protein dynamics where adsorbed water molecules per protein at charged and polar groups is shown to have a strong influence on protein flexibility, perhaps due to charge screening effect [2,5]. Many other protein properties become identical to those in dilute solutions with hydration above $h = 0.2$. The specific volume of dried proteins is 3%–7.5% larger than the value in solutions, primarily due to the electrostriction about the charged groups [6,35]. Such difference disappears as the hydration level increases to $h = 0.2$ [2,36]. The protein conformation is shown to be similar in the dry state and in solution to a resolution ~0.1 nm [2]. A Raman study showed that the conformation and flexibility changes only at low water content within a range of $h = 0.1$–0.2 [37]. Above $h \sim 0.17$, those properties become similar to those in solutions. These facts suggest that, even if there is possible partial unfolding or conformational change during the dehydration or rehydration process, it is reversible and occurs at a hydration level lower than what is needed for the upswing in water uptake [5,6,37].

E. The nature of upswing in water uptake

Using the linear region in the isotherms ($P/P_0 < 0.7$), we can estimate the amount of water adsorbed on charged and polar groups N_{polar} versus P/P_0 as shown in Fig. 3. Subtracting N_{polar} at P/P_0 from the total number of absorbed water molecules per protein, $N_{\text{hydration}}$, the excess water $N_{(\text{el})} = N_{\text{hydration}} - N_{\text{polar}}$ is obtained, as illustrated in Fig. 3. According to the modified Flory-Huggins theory, water molecules associated with $N_{(\text{el})}$ are mixed with protein structures such as side chains and depend on the elastic energy. The temperature dependence of K is essential in modulating the ratio of the elastic contribution to the chemical potential. The ratio could be much smaller at higher temperatures, and $\Delta H_{(\text{el})}$ will be too small to have a significant effect on hydration [25]. As a result, $N^0_{(\text{el})}$, the maximum of $N_{(\text{el})}$ reached at $P/P_0 = 1$, varies greatly with temperature. As shown in Table I, it is 185 out of $N^0_{\text{hydration}} = 360$ ($N_{\text{hydration}}$ at $P/P_0 = 1$) at 18 °C, whereas it is only 23 out of 167 at 2 °C. The inset of Fig. 3 plots $N^0_{(\text{el})}$ versus temperature. $N^0_{(\text{el})}$ decreases gradually from 18 to 2 °C. It is important to note that simulations based on surface adsorption model require about six layers to produce the upswing in water uptake shown in Fig. 3 [25]. However, the number of absorbed water molecules per protein $N^0_{\text{hydration}} = 360$ at 18 °C is smaller than the number needed for the monolayer coverage of the protein surface based on lysozyme structure [2]. Therefore, water molecules associated with $N_{(\text{el})}$ should be considered as incorporated in the protein structure with important consequences for protein structures and dynamics.

F. Molecular dynamics simulations under partially and fully hydrated conditions

The elastic modulus K at high hydration level is an averaged macroscopic measure of the microscopically heterogeneous flexibility of the protein and is closely related to structural fluctuations [10,38–40]. They can be affected significantly by hydration and temperature [31,34,41–43]. Therefore the molecular dynamics simulations on lysozyme under partially and fully hydrated proteins may provide useful insights on the mechanism of hydration.

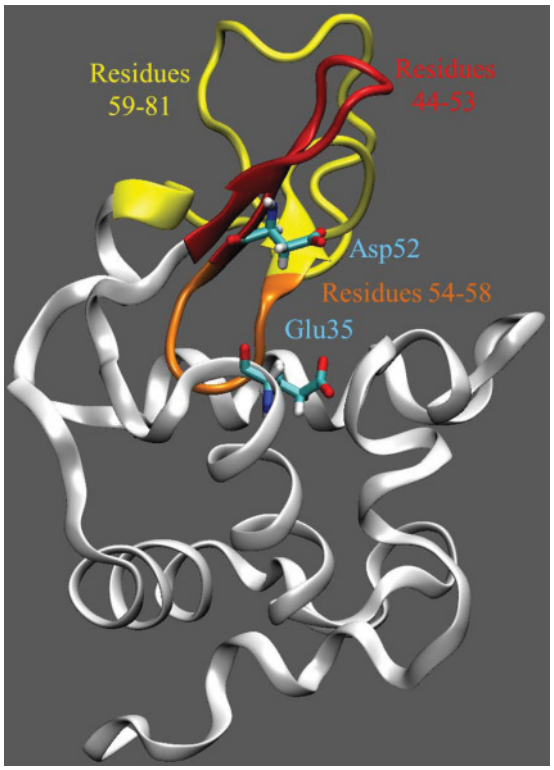

FIG. 5. (Color online) A ribbon representation of lysozyme structure from an averaged structure from the last 5 ns of 11-ns *NPT* simulations at 18 °C and $P/P_0 = 1$. The residues with elevated fluctuations and correlated motions by hydration water are highlighted in red (dark gray) (residues 44–53) and in yellow (light gray) (residues 59–81). The residues with low fluctuations in the β domain are shown in orange (gray) (residues 54–58). Residues that are essential for the completion of the enzymatic reaction (Glu35 and Asp52) are shown in an atomic representation. The rest of the residues are shown in a narrow ribbon representation.

The structure of HEWL has been solved to an exceptionally high resolution of 0.94 Å [14], exhibiting a typical globular protein fold with two main domains; a relatively more stable α domain consisting of residues 1–39 and 89–129 forming four α helices (helices A–D) and a 3_{10} helix, and a relatively more mobile β domain consisting of residues 40–88 forming three antiparallel β strands and another 3_{10} helix. Figure 5 shows a HEWL structure from molecular dynamics simulations. The enzymatic activity site is the cleft between the two domains, where a hexasaccharide can bind with a distortional stress at the fourth sugar, accelerating the cleavage of its glycosidic bond. Residues Glu35 and Asp52 on the opposing faces of the cleft and a water molecule are critically involved in the enzymatic reaction [44].

We performed molecular dynamics (MD) simulations on lysozyme [Protein Data Bank (PDB): 1IEE] [14] using AMBER 10.0 [45] with AMBER FF99SB force field at constant number of molecules, pressure, and temperature of $T = 291$ K (18 °C). Two systems with different lysozyme hydration

conditions were prepared for simulations. The experimentally determined numbers of hydration water molecules $N_{\text{hydration}}$ of 124 and 360 were used for the partially and fully hydrated systems, corresponding to the water vapor pressures of 12 mbar ($P/P_0 = 0.6$) and 20 mbar ($P/P_0 = 1$), respectively. Both systems were simulated for 11 ns with a time step of 2 fs. A cutoff of 10 Å was used for the Lennard-Jones interaction. The particle-mesh Ewald method was used for electrostatic interactions. Isotropic position scaling was used to maintain the pressure with a relaxation time of 2 ps. Langevin dynamics were used to control the temperatures using a collision frequency of 1.0 ps^{-1}. A periodic boundary condition was applied to both systems. The data were saved every 2 ps over the course of 11-ns simulation. The simulation results were analyzed using AMBER TOOLS [45] and visualized using Visual Molecular Dynamics (VMD) [46].

The MD simulations suggested that an increase in $N_{(el)}$ is associated with an increase in protein fluctuations and correlated motions. The root-mean-square fluctuation was calculated for both systems using the trajectories over the course of the last 5-ns simulations. Lysozyme showed greater flexibility under the fully hydrated condition, under which $N_{(el)}$ increased substantially. In addition, the structures averaged over the last 5-ns simulations were subjected to normal mode analyses using the Gaussian network model (GNM) [47,48]. As shown in Fig. 6, the overall level of fluctuation is lower when $N_{\text{hydration}} = 124$ [diagonal intensities in Fig. 6(a)] than when $N_{\text{hydration}} = 360$ [diagonal intensities in Fig. 6(b)]. In addition to the elevated-mean-square fluctuation at individual residues, it is also interesting to note that a high level of hydration greatly increases the correlated motions [off-diagonal intensities in Fig. 6(b)] of the β domain. This is most profound in residues 44–53 and residues 59–81, as shown from the diagonal intensities (in dashed squares) and off-diagonal intensities (in dashed rectangles) in Fig. 6. Residues 44–53 correspond to the $\beta 1$ and $\beta 2$ along with the linker as highlighted in red in Fig. 5, and are within the upper left dashed squares in Fig. 6. Residues 59–81 correspond to the large flexible loop hovering above the activity cleft as highlighted in yellow in Fig. 5, and are within the lower right dashed squares in Fig. 6. A noticeable exception is the absence of long-range correlated motions in residues 54–58 with the rest of the β domain as shown in orange in Fig. 5. A close examination of water distribution in the high-resolution crystal structure suggests that no water molecule can be found within the first hydration layer, which peaks at ~2.7 Å [49], near the segment of residues 54–58. In contrast, at least 23 water molecules can be found in the crystal structure covering the surface of other loops in the β domain. For lysozyme, the ability for the contour of the long cleft to be flexible is critical for the two-step enzymatic reaction [44]. The protein dynamics at the loop and hinge regions outside the active site have been found to be crucial to the motion of the two dynamically distinct domains and critical to enzymatic activities [39]. The presence of water molecules is essential to bringing about the modes of protein motion that would be otherwise unrealizable in the absence of water. The increase in protein fluctuations at higher hydration level suggests that hydration promotes protein fluctuations. Protein fluctuations are related to the elastic modulus at high hydration level through the equipartition theorem [10,40]. The elastic energy could become significant as the elastic modulus increases at lower temperature.

IV. CONCLUSIONS

The current study reveals the strong temperature dependence of the water uptake in lysozyme at high relative pressure below 10 °C. Such temperature dependence could be explained by the enhanced cost in the elastic energy upon hydration at lower temperature. The temperature dependence

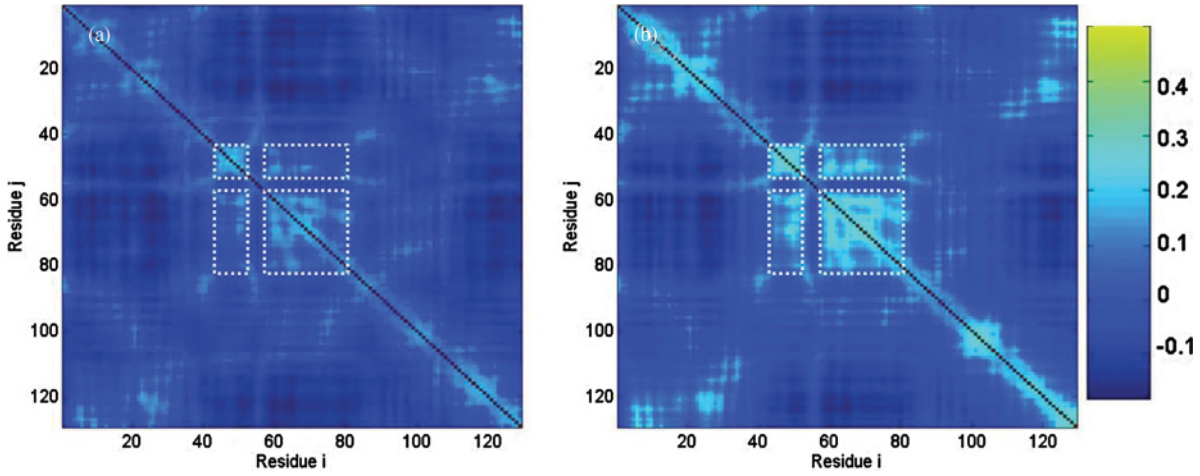

FIG. 6. (Color online) All-mode correlation plots of hen egg-white lysozyme from normal mode analysis using the Gaussian network model. The *NPT* simulations are under comparable conditions of the experiments at relative vapor pressure of (a) $P/P_0 = 0.6$ ($N_{\text{hydration}} = 124$) and (b) $P/P_0 = 1$ ($N_{\text{hydration}} = 360$) and a temperature of 18 °C. Averaged structures from the last 5 ns of 11-ns MD simulations were used for the GNM analyses. Cross correlation of motion is color-coded using the color (gray) scale to the right. The most profound increases in diagonal intensities are from residues 44–53 [upper left dashed squares and the red (dark gray) region in Fig. 5] and residues 59–81 [lower right dashed squares and the yellow (light gray) region in Fig. 5]. The corresponding increases in off-diagonal intensities between these two residue groups are labeled by rectangles.

of the elastic modulus K of the protein is responsible for the observed temperature dependence of the upswing in water uptake. Although related to the microscopic flexibility of the protein, the elastic modulus K used in our analysis of elastic energy is a parameter at the level of thermodynamics, not a model-dependent quantity at the molecular level or based on statistical mechanics. The elastic modulus K derived from water isotherms is consistent with the values of Young's modulus measured directly by mechanical measurement. This provides an independent validation of the solution picture. In the surface adsorption picture, the protein is simplified as a static surface with adsorption sites [23]. There is a clear boundary between water and proteins [27] and changes of protein structure upon hydration are not taken into account. In the solution picture, water mixes with proteins changing both the enthalpy and entropy of the protein-water system. Side chain rearrangements and volume expansion of protein upon hydration have an important effect on the enthalpy and entropy of the system [6]. Consequently, water molecules associated with the upswing in water uptake significantly alter the thermodynamic properties and could play a major role in determining the energy landscape and dynamics of the combined protein-water system [4]. The present work also shows that a decreased elastic modulus K is essential for enabling the sorption of water associated with the upswing in water uptake above $P/P_0 = 0.7$. Previous studies show that water adsorption on charged and polar groups reduces K [34] and enhances the protein flexibility [2]. This suggests that adsorbed water at charged and polar groups is an enabling factor for the occurrence of the upswing in water uptake at higher relative pressures. The interplay of elastic modulus and water absorption has an important role in determining the fluctuations of proteins and may be important for the functions of proteins such as enzymatic activities [39].

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