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Gibbs energies, enthalpies, and entropies of water and lysozyme at the inner edge of excess hydration

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The aim of this study is to simultaneously monitor the excess partial Gibbs energies, enthalpies, and entropies of water and white egg lysozyme and demonstrate how these quantities correlate with the coverage of the protein macromolecules by water molecules. Isothermal calorimetry and water sorption measurements were applied to characterize the hydration dependencies of the excess thermodynamic functions. The excess partial quantities are found to be sensitive to changes in the water and protein states. At the lowest water weight fractions (w_1), changes in the excess functions are primarily attributable to the addition of water. The transition of lysozyme from a glassy (rigid) to a flexible (elastic) state is accompanied by significant changes in the excess partial quantities. When the charged groups on the protein are covered, this transition occurs at $w_1 = 0.05$; when the coverage of both polar and weakly interacting surface elements is complete, the excess partial quantities become hydrated at $w_1 > 0.5$. At the highest water content, water addition has no significant effect on the excess quantities. At $w_1 > 0.5$, changes in the excess functions solely reflect changes in the state of the protein. © *2013 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4818527]

I. INTRODUCTION

Proteins are one of the most important classes of biological molecules. Water binding (hydration or biological water) plays a crucial role in determining the structure, stability, and functions of proteins.[1–8] However, there are essential differences between hydration and bulk water surrounding a protein.[1–5,9,10] This means that a characterization of the hydration of protein macromolecules requires elucidating the effects of both the protein on water and *vice versa*. Therefore, a quantitative estimation of the protein and water contributions to the thermodynamic functions of binary protein-water systems is of considerable importance and practical interest. By studying the thermodynamics of protein hydration, we can determine the resultant effects in terms of enthalpy, entropy, and Gibbs free energy.

Biothermodynamic studies have traditionally been of great importance in achieving a better understanding of protein-water interactions. Below, a short review of the available studies on the hydration of proteins is given. More comprehensive reviews are given in Refs. 1–4.

The partial Gibbs energy of mixing water with a protein is related to the water activity, a_w. This quantity can easily be measured experimentally from water sorption isotherms. Sorption isotherms (water content as a function of water activity) of many proteins have been reliably measured.[1–3,11–21] As is typical of proteins, the sorption isotherm has a sigmoid shape. The partial molar Gibbs energies of the mixing of water with lysozyme were estimated in the water weight fraction from 0 to 0.35.[16,21] It was shown that, in the studied range, the partial Gibbs energies of water were negative. They decreased with increasing water content and were close to zero at a water weight fraction (w_1) of 0.35.

Foss and Reyerson studied the water sorption by ribonuclease in the range of 0%–8% (g g^{-1}).[22] From the temperature dependence of the water sorption isotherms in the range 20–30 °C, they calculated differential entropies of water sorption. Luscher-Mattli and Ruegg[21,23] calculated the entropies of water sorption by lysozyme and α-chymotrypsin. They studied the water sorption isotherms in the water weight fraction range of 0 to ∼0.4. The hydration entropies were calculated from the temperature dependence of the water vapor pressure in the range of 10–40 °C. Bone studied the water sorption by lysozyme in the range of 1.5%–19% (g g^{-1}).[14] Calculations were performed using the temperature dependence of the water vapor pressure in the range of 6–46 °C. Sorption calorimetry has been used to measure the absorption isotherm of water by lysozyme and the corresponding hydration entropies in a water weight fraction of 0–0.35.[16] The hydration entropies vary strongly with the hydration level. They are large and negative at low hydration levels. As the water content increases, the hydration entropies approach the entropy of condensation of pure water.

Isothermal calorimetry is one of the most effective methods for obtaining reliable thermochemical information about the interactions of proteins with water in various environments. Amberg measured the heat of water vapor sorption using bovine serum albumin within a water content range of 0%–12% (g g^{-1}) at 20 °C.[24] The enthalpies of the solution of the dried and hydrated ribonuclease A (RNase A) were

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measured using isothermal calorimetry at 25 °C.[25] The water content of RNase A was varied from 0% to 26% (g g^{-1}), and the enthalpy of the solution was negative (exothermic). As the water content of RNase A increases, the enthalpy of the solution approaches zero. Smith *et al.* calorimetrically measured the heat of water adsorption by lysozyme in the range of relative water vapor pressures from 0 to 0.895[26] and obtained both the sorption isotherm and the enthalpy of hydration of the protein within a water content range of 0%–18% (g g^{-1}) at 25 °C. Pineri *et al.*[27] have measured both the water sorption-desorption isotherms and the enthalpy of water sorption-desorption for collagen; the most important conclusion from this study is the evidence that different states of water sorption correspond to different energies. Sorption calorimetry has been used to measure the adsorption isotherm of water by lysozyme and the corresponding heat effects in the entire range of water activities.[16] Our research group has developed an experimental method for measuring the heat effects of the hydration-dehydration of proteins over the entire range of water content.[17,18] The interaction enthalpy depends significantly on the initial water content and hydration history.

The obtained water sorption isotherms, enthalpies, and entropies[11–27] contain complete information about the binary water-protein systems, including the corresponding conformational changes in the protein structure and glass transition. However, the simultaneous estimation of the protein and water contributions to the Gibbs energy, enthalpy, and entropy of binary protein-water systems in the entire range of water content has not yet been attempted.

The thermodynamic functions of other proteins have been similarly studied. For example, the thermodynamic properties of BPTI (bovine pancreatic trypsin inhibitor) were assessed using a molecular dynamics simulation and normal-mode analysis.[28] The partial internal energy and Helmholtz energy of BPTI and water have only been computed for the dry and fully hydrated protein.[28] The apparent heat capacities of lysozyme[29] and BPTI[28] were calculated in the water content range from the dried protein to the fully hydrated limit. However, the apparent heat capacity of water was only estimated for the dry and hydrated protein states.

A novel methodology has recently been proposed for simultaneously estimating the excess partial quantities of water and proteins.[9,10] This method is based on the analysis of the thermodynamic functions of mixing. In the present study, we applied this method to simultaneously monitor the excess partial Gibbs energies, enthalpies, and entropies of water and protein in the entire range of water content. The aim of this study was to determine the excess partial Gibbs energies, enthalpies, and entropies of water and protein, and demonstrate how these quantities correlate with the coverage of the protein macromolecules by water molecules.

White egg lysozyme was used as a model. This protein is one of the most well-studied and applied in biopolymer investigations.[30,31] Lysozyme is a small monomeric protein of 129 amino acid residues, containing no non-protein components. The physiological role of lysozyme is to hydrolyze polysaccharide chains.[30,31]

II. METHODOLOGY

A. Excess partial quantities

The thermodynamic properties of a real binary water-protein system can be expressed in terms of the excess functions, Z^E,[32–34] i.e., the difference between the observed thermodynamic function of mixing, Z^m, and the function for an ideal binary mixture, Z^m_{id}:

$$Z^E = Z^m - Z^m_{id}. \tag{1}$$

The Z^m_{id} values can be calculated using Eq. (2):

$$Z^m_{id} = w_1 Z^0_1 + w_2 Z^0_2, \tag{2}$$

where Z^0_1 and Z^0_2 are the thermodynamic function values for pure water and pure protein, and w_1 and w_2 are the mass fractions of water and protein, respectively.

Deviations of the excess functions from zero indicate the extent to which the studied binary system is non-ideal due to strong specific interactions between components (i.e., hydrogen bonding and charge-charge interactions).

The Z^E values are composed of two components:

$$Z^E = w_1 \bar{Z}^E_1 + w_2 \bar{Z}^E_2, \tag{3}$$

where $\bar{Z}^E_1$ is the excess partial function for component 1 (water), and $\bar{Z}^E_2$ is the excess partial function for component 2 (protein).

B. Excess partial Gibbs energies

The Gibbs-Duhem equation ($\sum_i n_i d\mu_i = 0$) was applied to calculate the excess Gibbs free energies. For a binary mixture, the Gibbs-Duhem equation can be described using Eq. (4):

$$n_1 d\mu_1 + n_2 d\mu_2 = 0, \tag{4}$$

where μ_1 and μ_2 are the partial Gibbs energies of water and the protein, respectively.

This implies that if one partial quantity increases, the other must decrease:

$$d\mu_2 = -\frac{n_1}{n_2} d\mu_1 = -\frac{x_1}{x_2} d\mu_1, \tag{5}$$

where x_1 and x_2 are the water and protein mole fractions in the water-protein mixtures, respectively.

At constant pressure and temperature:

$$d\mu_i = RT d(\ln a_i), \tag{6}$$

where a_i is the water or protein activity in the water-protein mixtures.

Equation (5) can be transformed into Eq. (6):

$$d(\ln a_2) = -\frac{1 - x_2}{x_2} d(\ln a_1). \tag{7}$$

The water and protein activity coefficients, γ_i, are calculated using Eq. (8):

$$a_i = \gamma_i * x_i, \tag{8}$$

TABLE I. Symbols used in Sec. II.

Symbol	Unit	Name
w	...	Water
P	...	Protein
h	...	Hydrated
m_w^h	[g water]	Amount of water bound to the protein
m_P	[g protein]	Amount of the protein
m_w^{sol}	[g water]	Amount of water bound to the protein in the diluted aqueous solution
W_1	...	Weight fraction of water
W_2	...	Weight fraction of protein
$\overline{H}_{w,.liquid}$	[J g^{-1} water]	Partial enthalpy of water in pure liquid state at 25 °C and atmospheric pressure
$\overline{H}_{w,.protein}$	[J g^{-1} water]	Partial enthalpy of water bound to the protein at 25 °C and atmospheric pressure
$\overline{H}_P^0$	[J g^{-1} protein]	Partial enthalpy of the dried protein at 25 °C and atmospheric pressure
$\overline{H}_P^h$	[J g^{-1} protein]	Partial enthalpy of the hydrated protein at 25 °C and atmospheric pressure
$\overline{H}_{w,..protein}^{sol}$	[J g^{-1} water]	Partial enthalpy of water bound to the protein in the diluted aqueous solution
$\overline{H}_P^{sol}$	[J g^{-1} protein]	Partial enthalpy of the protein in the diluted aqueous solution
$\Delta_{sol}H$(dried)	[J]	Enthalpy of solution of the dried protein in pure water at 25 °C and atmospheric pressure
$\Delta_{sol}H$(hydrated)	[J]	Enthalpy of solution of the hydrated protein in pure water at 25 °C and atmospheric pressure

where x_i is the water or protein mole fraction in the water-protein mixtures and γ_i is the water or protein activity coefficient in the water-protein mixtures.

For non-ideal systems, Eq. (7) can be transformed into Eq. (9):

$$d(\ln\gamma_2) + d(\ln x_2) = -\frac{1-x_2}{x_2}d(\ln\gamma_1) - \frac{1-x_2}{x_2}d(\ln(1-x_2)). \quad (9)$$

For ideal systems, Eq. (7) can be transformed into Eq. (10):

$$d(\ln x_2) = -\frac{1-x_2}{x_2}d(\ln(1-x_2)). \quad (10)$$

The difference between Eq. (9) and (10) is

$$d(\ln\gamma_2) = -\frac{1-x_2}{x_2}d(\ln\gamma_1). \quad (11)$$

The integration of Eq. (11) from $\ln(\gamma_1^0)$ at $x_2 = 1$ to $\ln(\gamma_1)$ at x_2^* gives Eq. (12):

$$\ln\gamma_2 = -\int_{\ln(\gamma_1^0)}^{\ln(\gamma_1)} \frac{1-x_2}{x_2}d(\ln\gamma_1) = -\int_{\ln(\gamma_1^0)}^{\ln(\gamma_1)} \frac{n_1}{n_2}d(\ln\gamma_1). \quad (12)$$

The excess partial Gibbs energy of water and protein can be calculated using Eqs. (13) and (14):

$$\mu_2^E = -\int_{-\infty}^{\mu_1^E} \frac{n_1 * M_1}{n_2 * M_2}d(\mu_1^E) = -\int_{-\infty}^{\mu_1^E} \frac{w_1}{w_2}d(\mu_1^E), \quad (13)$$

$$\mu_1^E = \frac{RT}{M_1}\ln\gamma_1, \quad (14)$$

where μ_1^E (J/g water) is the excess partial Gibbs energy of water; μ_2^E (J/g protein) is the excess partial Gibbs energy of protein; w_1 and w_2 are the weight fractions of water and protein, respectively; M_1 is the molecular mass of water; and M_2 is the molecular mass of lysozyme. (Table I)

The excess Gibbs energy of mixing (J/g mixture) can be calculated using Eq. (15):

$$G^E = w_1\mu_1^E + w_2\mu_2^E. \quad (15)$$

C. Excess partial enthalpies

The excess partial enthalpies of the protein and water were calculated as described previously.[9] This method facilitates the estimation of the individual protein and water partial quantities in the entire range of water content. The interaction enthalpy observed during the addition of water to the protein is considered to be an excess quantity of the mixing of water with the protein (H^E, Eq. (3)). The H^E value is calculated from the enthalpies of the solution of the dried and hydrated protein in pure liquid water. The enthalpy of the solution of the dried protein in pure liquid water, $\Delta_{sol}H$(dried), is described in Eq. (16). The final state of the protein (Eq. (16) and Eq. (17)) is a diluted solution in water at 25 °C and at atmospheric pressure:

$$\Delta_{sol}H(\text{dried}) = \left[\overline{H}_P^{sol}m_P\right] - \left[\overline{H}_P^{o}m_P\right] + \left[\overline{H}_{w,.protein}^{sol}m_w^{sol}\right] - \left[\overline{H}_{w,..liquid}m_w^{sol}\right], \quad (16)$$

where $\overline{H}_P^0$ is the partial enthalpy of the dried protein at 25 °C and at atmospheric pressure; $\overline{H}_{w,.liquid}$ is the partial enthalpy of water in pure liquid state at 25 °C and at atmospheric pressure; $\overline{H}_{w,.protein}^{sol}$ is the partial enthalpy of water bound to the protein in the diluted aqueous solution; $\overline{H}_P^{sol}$ is the partial enthalpy of the protein in the solution; m_P is the amount of the protein; m_w^h is the amount of water bound to the protein; and m_w^{sol} is the amount of water bound to the protein in the diluted aqueous solution.

The enthalpy of solution of the initially hydrated protein in pure liquid water, $\Delta_{sol}H$(hydrated), is described

in Eq. (17):

$$\Delta_{sol}H(\text{hydrated}) = \left[\overline{H}_P^{sol} m_P\right] - \left[\overline{H}_P^{h} m_P\right] + \left[\overline{H}_{w,.protein}^{sol} m_w^{sol}\right] - \left[\overline{H}_{w,..liquid} m_w^{sol}\right] + \left[\overline{H}_{w,..liquid} m_w^{h}\right] - \left[\overline{H}_{w,.protein}^{h} m_w^{h}\right], \quad (17)$$

where $\overline{H}_{w,.protein}^{h}$ is the partial enthalpy of water bound to the protein and $\overline{H}_P^{h}$ is the partial enthalpy of the hydrated protein.

As shown in Eq. (18), the H^E value is calculated from the enthalpies of solution of the dried, $\Delta_{sol}H$(dried), and hydrated, $\Delta_{sol}H$(hydrated), protein in water, measured using isothermal calorimetry:

$$\frac{\Delta_{sol}H(\text{dried}) - \Delta_{sol}H(\text{hydrated})}{m_w^h + m_P} = \left[\overline{H_P^h} - \overline{H_P^0}\right]\frac{m_P}{m_P + m_w^h} + \left[\overline{H}_{w,.protein}^{h} - \overline{H}_{w,.liquid}\right]\frac{m_w^h}{m_P + m_w^h} = \left[\overline{H_P^h} - \overline{H_P^0}\right]w_2 + \left[\overline{H}_{w,.protein}^{h} - \overline{H}_{w,.liquid}\right]w_1 = H^E. \quad (18)$$

The excess partial enthalpies of the protein and water are calculated using Eqs. (19) and (20):

$$\bar{Z}_1^E = Z^E - w_2\left(\frac{\partial Z^E}{\partial w_2}\right)_{T,P} = [\overline{H}_{w,.protein} - \overline{H}_{w,..liquid}] = \overline{H}_1^E, \quad (19)$$

$$\bar{Z}_2^E = Z^E - w_1\left(\frac{\partial Z^E}{\partial w_1}\right)_{T,P} = \overline{\left[H_P^h - H_P^0\right]} = \overline{H}_2^E, \quad (20)$$

where $\overline{H}_1^E$ and $\overline{H}_2^E$ are the excess partial enthalpies of the water and protein, respectively, and w_1 and w_2 are the mass fractions of water and protein, respectively.

D. Excess partial entropies

The excess partial entropies are calculated using Eqs. (21)–(23):

$$\frac{H^E - G^E}{T} = S^E, \quad (21)$$

$$\overline{S}_1^E = S^E - w_2\left(\frac{\partial S^E}{\partial w_2}\right)_{P,T}, \quad (22)$$

$$\overline{S}_2^E = S^E - w_1\left(\frac{\partial S^E}{\partial w_1}\right)_{P,T}, \quad (23)$$

where $\overline{S}_1^E$ and $\overline{S}_2^E$ are the excess partial entropies of water and protein.

III. EXPERIMENTAL

A. Materials

Hen egg-white lysozyme of the highest commercially available purity was purchased from Sigma Chemical (St. Louis, MO) and used without further purification. The purity of the protein samples was verified through electrophoresis and dynamic light-scattering measurements (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, USA) to be more than 95%. The molecular weight of the protein was taken as 14 300 Da. The water used was doubly distilled.

B. Water sorption measurements

The dried protein preparation (zero hydration level) was obtained through vacuum drying using a microthermoanalyzer "Setaram" MGDTD-17S (±0.00001 g) at 25 °C and 0.1 Pa, until a constant sample weight was reached. The water content of the dried protein was estimated as 0.003 ± 0.002 g water g^{-1} protein using the Karl Fischer titration method, according to the recommendations.[35] This zero hydration level was described using $\overline{H}_P^0$ (Eqs. (16)–(18)).

At the lowest and intermediate water weight fractions, the protein preparations were prepared as follows. The initially dehydrated protein samples were exposed to pure water vapor. The water vapor was consecutively flowed through a thermostated glass tube with drying agent (P_2O_5), a thermostated saturator filled with saturated salt solution, and a cell containing the protein sample. The schematic representation of the experimental setup is given in Fig. 1. An external ethylene glycol thermostat (RC 6 from Lauda, Germany) was used to control the temperature with a precision of 0.1 °C. Sorption equilibrium was reached after 180 min. The water activity (a_w) in the vapor phase was adjusted by changing the saturated salt solution in the saturator. The water activities of the saturated salt solutions were taken from Refs. 36 and 37. The following salts were used: LiBr (a_w = 0.064), KOH (a_w = 0.078), LiCl (a_w = 0.11), $CaBr_2$ (a_w = 0.17), CH_3COOK (a_w = 0.22), $MgCl_2$ (a_w = 0.33), K_2CO_3 (a_w = 0.44), $Mg(NO_3)_2$ (a_w = 0.53), NaCl (a_w = 0.75), KCl (a_w = 0.84), KNO_3 (a_w = 0.94), and K_2SO_4 (a_w = 0.97). The salts used for sample conditioning were of analytical pure grade. The water content of the samples after equilibration was measured by drying under vacuum using a microthermoanalyzer "Setaram" MGDTD-17S at 25 °C and 0.1 Pa, until a constant sample weight was reached.

At the highest water weight fractions (a_w > 0.98), the water activity was adjusted by changing the unsaturated NaCl-

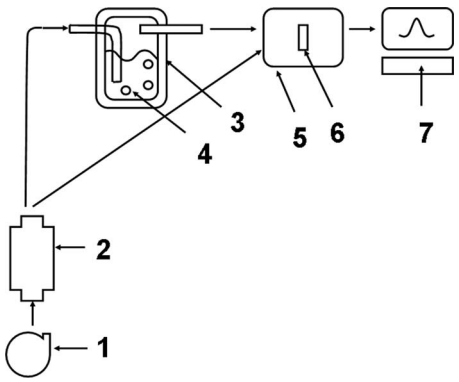

FIG. 1. Schematic representation of the experimental setup of hydration measurements. The components of the experimental setup: 1—pump; 2—thermostated glass tube with P_2O_5; 3—thermostated saturator; 4—pure liquid water or salt solutions; 5—thermostated cell; 6—protein sample; 7—microthermoanalyzer "Setaram" MGDTD-17S.

water solutions at 25 °C. The a_w values of the NaCl-water solutions were taken from Ref. 38. This technique was used for the protein-water systems for $w_1 > 0.6$.

C. Calorimetry

The enthalpies of the solution of the dried, $\Delta_{sol}H$(dried), and hydrated, $\Delta_{sol}H$(hydrated), protein in pure liquid water (Eqs. (16) and (17)) were measured at 25 °C using a Setaram BT-2.15 calorimeter, according to a previously described procedure.[39,40] Briefly, 8–10 mg of protein was prepared in 4.0 ml of water in the calorimetric cell. A typical duration of a calorimetric experiment lasted approximately 40 min. The typical heat evolution curve recorded from the dissolution of a solid protein in pure liquid water has been previously described.[41]

The calorimeter was calibrated using the Joule effect and tested by measuring the solution enthalpy of potassium chloride in water. Potassium chloride of 99.9% purity was recrystallized and dried as previously described.[42] The measured value of the solution enthalpy, $\Delta_{sol}H^{KCl/H_2O}$ (25 °C, $m = 0.0277$ mol kg^{-1}) = 17.43 ± 0.08 kJ mol^{-1}, is consistent with the recommendations of the ICTAC working group "thermochemistry:"[43] $\Delta_{sol}H^{KCl/H_2O}$ (25 °C, $m = 0.02775$ mol kg^{-1}) = 17.47 ± 0.07 kJ mol^{-1}.

The dried protein preparation (zero hydration level) was obtained through vacuum drying using a microthermoanalyzer "Setaram" MGDTD-17S at 25 °C and 0.1 Pa, until a constant sample weight was reached. This zero hydration level was described using the $\overline{H}_P^0$ values (Eqs. (16)–(18)).

At the lowest and intermediate water content, the protein preparations were prepared as described in Sec. II B (Fig. 1). At the highest water content, the protein and water samples were mixed in the calorimetric cell at various water weight fractions and at 25 °C. The masses of the protein samples ranged from 8 to 10 mg. The water content of the samples after equilibration was measured through vacuum drying using a microthermoanalyzer "Setaram" MGDTD-17S at 25 °C and 0.1 Pa, until a constant sample weight was reached.

The hydrated protein samples were described using $\overline{H}_P^h$ and $\overline{H}_{w,.protein}$ (Eqs. (17)–(20)).

IV. RESULTS AND DISCUSSION

Fig. 2 shows the dependency of the μ_1^E values on the hydration level of lysozyme. The μ_1^E values were calculated using Eq. (14). As shown in Fig. 2, the μ_1^E values are consistent with previously published results,[13] indicating that the apparatus and the experimental procedure are adequate to calculate the thermodynamic functions. Fig. 3 presents the μ_2^E values as a function of the weight fraction of water. The μ_2^E values were calculated using Eq. (13).

Figs. 4(a)–4(c) show the dependencies of the G^E, H^E, and TS^E values (excess functions of mixing per-unit-mass of the water-protein mixture) on the hydration level of lysozyme. The G^E, H^E, and TS^E values were estimated using Eqs. (15), (18), and (21).

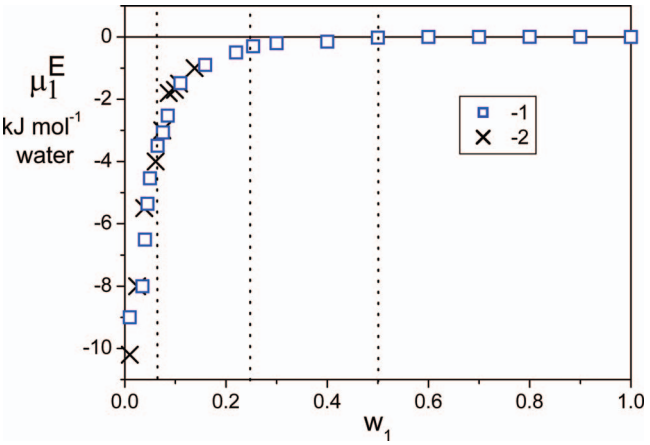

FIG. 2. Excess Gibbs energy of water, μ_1^E, as a function of the weight fraction of water, w_1, at 25 °C: (1) This study. Standard errors of estimation of the μ_1^E values were 0.2–0.3 kJ mol^{-1} water; (2) adapted data from Ref. 13.

In ideal binary mixtures (mixtures of two components, W [water] and P [protein]) the average W-P interactions are the same as the average W-W and P-P interactions. Non-ideal mixtures are composed of particles for which the W-W, P-P, and W-P interactions are all different. As shown in Figs. 4(a)–4(c), the G^E, H^E, and TS^E values differ significantly from zero, indicating that the water-protein mixtures are non-ideal in the entire range of water content. Figs. 4(a)–4(c) present the dependencies of the $w_i\bar{Z}_i^E$ ($w_1\mu_1^E$, $w_2\mu_2^E$, $w_1\overline{H}_1^E$, $w_2\overline{H}_2^E$, $w_1\overline{TS}_1^E$, and $w_2\overline{TS}_2^E$) functions plotted versus the water weight fraction. These functions were calculated using Eq. (3). They show the individual protein and water contributions to the excess thermodynamic functions in the entire range of water content. Figs. 4(a)–4(c) demonstrate that the protein contributions ($w_2\bar{Z}_2^E$) reach maximal values at $w_1 \sim 0.25$. However, the $w_1\bar{Z}_1^E$ functions reach maximal values at low water content ($w_1 \sim 0.05$).

Figs. 5 and 6 present the excess partial entropy of water, $\overline{TS}_1^E$, and the excess partial entropy of lysozyme, $\overline{TS}_2^E$, as functions of the weight fraction of water. These partial quantities were calculated using Eqs. (22) and (23).

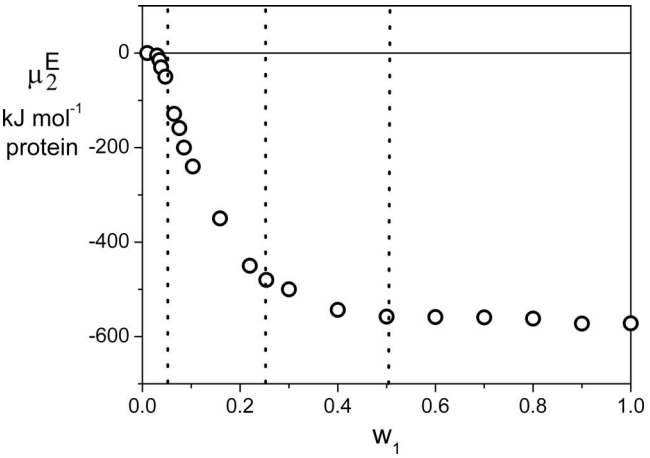

FIG. 3. Excess Gibbs energy of lysozyme, μ_2^E, as a function of the weight fraction of water, w_1, at 25 °C.

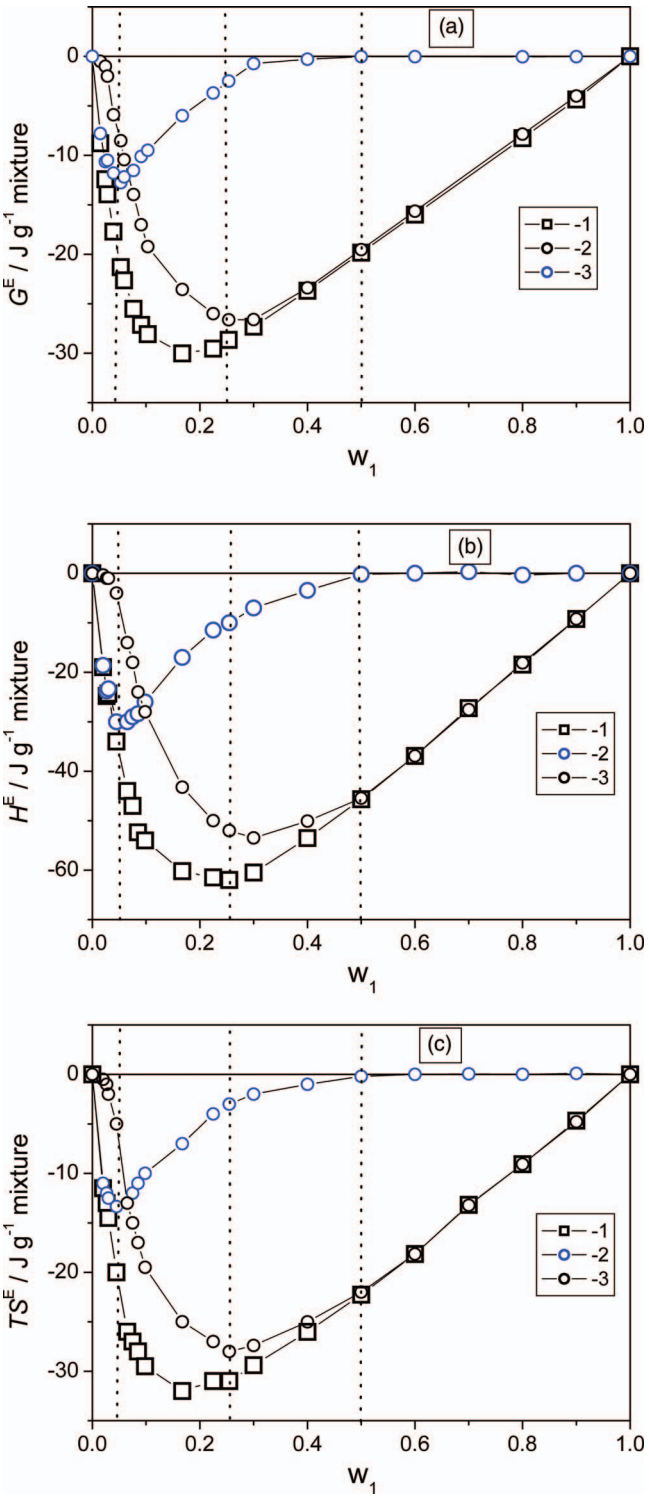

FIG. 4. Excess thermodynamic functions plotted versus the weight fraction of water, w_1, at 25 °C: (a) 1—G^E; 2—$w_1\mu_1^E$; 3—$w_2\mu_2^E$; (b) 1—H^E; 2—$w_1\overline{H}_1^E$; 3—$w_2\overline{H}_2^E$ (2), and (c) 1—TS^E; 2—$w_1\overline{TS}_1^E$; 3—$w_2\overline{TS}_2^E$.

To show the reliability of our experiments, the $\overline{TS}_1^E$ values (Fig. 5) were compared with previously published data. A similar monotonic curve for the hydration dependence of the water entropy was observed from the sorption calorimetry data (Fig. 5).[21] The isosteric entropies were estimated from the temperature dependence of the water sorption isotherm

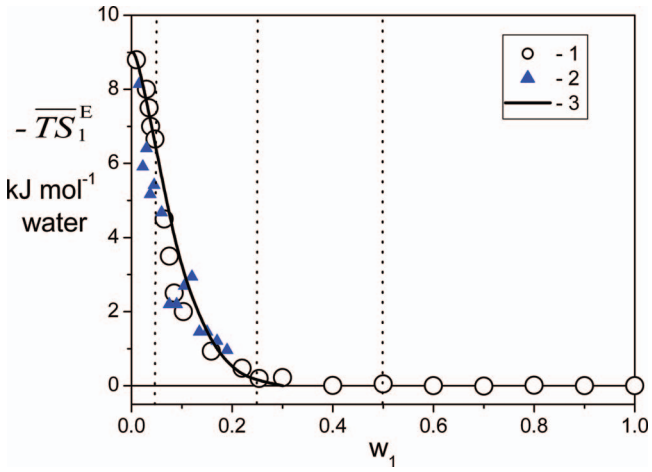

FIG. 5. Lysozyme. (1) Water entropy, $\overline{TS}_1^E$, as a function of the weight fraction of water, w_1, at 25 °C (this study). The reference state is pure liquid water at 25 °C and at atmospheric pressure. (2) Water entropy estimated from the temperature dependence of the water sorption isotherm (adapted data from Fig. 6 of Ref. 14). (3) Water entropy estimated using sorption calorimetry at 25 °C. Adapted data from Ref. 16.

(The van't Hoff plots [ln P versus 1/T] at various lysozyme water content [Fig. 6 from Ref. 14]). The isosteric quantity is the change in partial molar quantity for the transfer of water from the vapor phase to the protein at water equilibrium pressure P; these values were estimated using Eq. (24). The parameters of Eq. (24) are given in Table II. Equation (24) is applied for constant (isosteric) water content (Γ). As shown in Fig. 5, our data are consistent with previously published results.

$$\ln P_\Gamma = \frac{\Delta\overline{S}_\Gamma}{R} - \frac{\Delta\overline{H}_\Gamma}{R}\left(\frac{1}{T}\right) = \frac{\overline{S}_v - \overline{S}_P}{R} - \frac{\overline{H}_v - \overline{H}_P}{R}\left(\frac{1}{T}\right), \tag{24}$$

where $\overline{S}_v$ is the partial molar entropy of water in the vapor phase; $\overline{S}_P$ is the partial molar entropy of the sorbed water; $\overline{H}_v$ is the partial molar enthalpy of water in the vapor phase; $\overline{H}_P$ is the partial molar enthalpy of the sorbed water; and R is the gas constant.

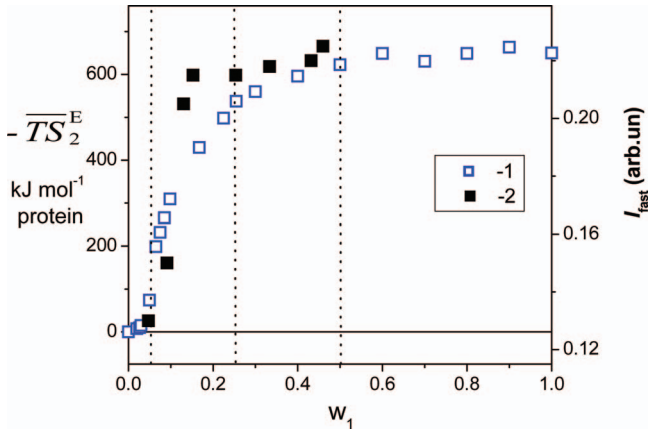

FIG. 6. (1) Excess partial entropy of lysozyme, $\overline{TS}_2^E$, as a function of the weight fraction of water, w_1, at 25 °C. (2) Hydration dependence of the integrated quasielastic scattering (QES) intensity of the fast process, I_{fast}, at 22 °C. Adapted data from Ref. 46.

TABLE II. Parameters of Eq. (24).[a]

No.	Water content, % (g g^{-1})	$\frac{\Delta\overline{S_\Gamma}}{R}$	$\frac{\Delta\overline{H_\Gamma}}{R}$	R	N	S_o
1	1.5	25.9 (0.8)	7980 (240)	0.997	9	0.097
2	2.25	25.0 (1.0)	7350 (140)	0.995	9	0.114
3	3.0	25.2 (0.5)	7410 (160)	0.998	9	0.064
4	3.75	24.7 (0.6)	6830 (160)	0.998	9	0.067
5	4.5	24.8 (0.7)	7050 (200)	0.997	9	0.080
6	6.0	24.5 (1.2)	6880 (350)	0.991	9	0.142
7	7.5	23.5 (0.4)	6400 (110)	0.999	9	0.045
8	9.0	23.5 (0.7)	6320 (210)	0.996	9	0.084
9	10.5	23.7 (0.4)	6310 (130)	0.999	8	0.041
10	12.0	23.8 (0.4)	6300 (110)	0.999	9	0.044
11	13.5	23.2 (0.4)	6070 (120)	0.999	9	0.050
12	15.0	23.2 (0.5)	6050 (140)	0.998	8	0.044
13	17.0	23.1 (0.8)	5970 (240)	0.998	5	0.038
14	19.0	23.0 (2.4)	5920 (680)	0.993	3	0.049

[a]N is the number of experimental points; S_o is the standard error of estimation; and R is the correlation coefficient.

The partial quantity curves presented in Figs. 2–6 can be divided into four parts.

Regime 1 ($w_1 = 0$–0.05). The μ_2^E, $\overline{TS}_2^E$, and $\overline{H}_2^E$ values are close to zero and do not depend on the water content (Figs. 3 and 6 [this study] and Fig. 3 from Ref. 9). At the lowest water content, the proteins are in a glassy (rigid) state.[1] No biological activity was observed at the lowest water content (Fig. 4 from Ref. 9). The fact that the dehydrated proteins are in the glassy state explains this feature of regime 1. The lysozyme excess partial quantities are close to zero, reflecting the fact that all of the protein molecules contact the same protein molecules during this range of water content.

At $w_1 < 0.05$, the $w_2\bar{Z}_2^E$ values are close to zero (Figs. 4(a)–4(c)). At $w_1 \sim 0.05$, the $w_1\bar{Z}_1^E$ functions reach maximal values. These facts imply that, at the lowest water content, changes in the excess thermodynamic functions solely reflect water addition.

The heat capacities of insulin and chymotrypsinogen A were examined in the temperature range of −263 to 37 °C.[44] No phase transition corresponding to the ice-liquid water transition was observed at low hydrations.

This region corresponds to the hydration of charged groups.[1,2,45] At low water content, the μ_1^E, $\overline{TS}_1^E$, and $\overline{H}_1^E$ values differ significantly from zero (Figs. 2 and 5 [this study], and Fig. 2 from Ref. 9). The μ_1^E, $\overline{H}_1^E$, and $\overline{TS}_1^E$ values are highly negative. As concluded in Ref. 9, the $\overline{H}_1^E$ ($w_1 = 0$) values are close to those observed for diethylamine, suggesting that, at the lowest w_1 values, the interaction with the protein elements containing the amino groups might be a dominant factor controlling the state of water molecules.

Regime 2 ($w_1 = 0.05$–0.25). Proteins undergo a glasslike dynamic transition at water content of approximately 10 wt.% at 25 °C.[1] This water content falls within regime 2 in the present study. The transition from the glassy (rigid) to the flexible (elastic) state is accompanied by significant changes in the properties of the proteins.[1] For example, during the isothermal sorption of water, a glasslike transition results in a step in the μ_2^E, $\overline{TS}_2^E$, and $\overline{H}_2^E$ curves (Figs. 2 and 6, and Fig. 3 from Ref. 9). The μ_2^E, $\overline{TS}_2^E$, and $\overline{H}_2^E$ values change sharply from extremely low values to highly negative values. The apparent heat capacity of lysozyme, ϕC_{p2}, determined from isothermal experiments using a drop calorimeter, increases from extremely low values to high values in this water content interval.[17]

Regime 2 corresponds to the hydration of polar groups.[1,2,45] In the water content range $w_1 = 0.05$–0.25, the $\overline{TS}_1^E$ values change sharply from highly negative (∼−9.0 kJ mol^{-1} water) to moderate (∼−2.0 kJ mol^{-1} water) values (Fig. 5). This sharp transition reflects the formation of a spanning hydrogen-bonded network of water.[2,5] The formation of this network occurs via a quasi two-dimensional percolation transition of the hydration water.[5]

Quasielastic neutron and light-scattering techniques were employed to characterize the effect of hydration on the internal dynamics of lysozyme in the picoseconds-to-nanoseconds time range.[46] The increased hydration activates the fast relaxation process in regime 2 (Fig. 6). Notably, the $\mu_1^E - w_1$, $\overline{TS}_1^E - w_1$, and $\overline{H}_1^E - w_1$ curves, and the dependence of fast conformational fluctuations on the hydration level, are similar to the hydration dependence of hydrogen isotope exchange[47] (Figs. 2 and 5 [this study], and Fig. 2 from Ref. 9).

Regime 3 ($w_1 = 0.25$–0.5). Regime 3 indicates the appearance of the "structured" water.[45] "Structured" water is composed of molecules that interact with hydrophobic surface patches on the protein-bridging between bound water molecules. Rupley and Careri[29] asserted that this region contains condensed water molecules over weakly interacting surface elements, which are likely nonpolar atoms, not polar groups or adjacent-charged.

The $\overline{TS}_1^E$ and $\overline{H}_1^E$ values alter, to a moderate degree, in this region of water content (Fig. 5 [this study] and Fig. 2 from Ref. 9). The $\overline{TS}_1^E$ values vary between ∼−1.0 and 0 kJ mol^{-1} water. This region is probably composed of the completion of the formation of the spanning hydrogen-bonded network of water.

Significant changes in the amide I and III regions of the dehydrated proteins were observed using Fourier transform infrared (FTIR) spectroscopy.[48–54] Klibanov and Griebenow[51] examined several proteins and showed that dehydration lowers the α-helix content and increases the β-sheet content. Using FTIR spectroscopy, Constantino *et al.*[51] demonstrated that the β-sheet content in the dehydrated lysozyme state was approximately 44%; in the aqueous solution, the β-sheet content was about 18%. The dehydration-induced alterations were essentially reversible.[51,54]

The dehydration-induced changes in the distribution of isotropic chemical shifts for lysozyme were obtained from the ^{13}C NMR spectra.[55,56] Solid-state ^{13}C-NMR spectra of lysozyme revealed that the dried protein is characterized by a relatively broad distribution of isotropic chemical shifts. Hydration reduces the distribution of the conformations that are sampled by the protein. The change in the distribution of conformational states begins at a hydration level of $w_1 = 0.09$–0.13 (regime 2). This change is largely complete

at a hydration of $w_1 > 0.15$ (regime 3). Changes in the μ_2^E, $\overline{TS}_2^E$, and $\overline{H}_2^E$ values are also complete within regime 3 (Figs. 3 and 6 [this study] and Fig. 3 from Ref. 9).

The onset of biological activity was observed in this region. Rupley *et al.*[57] examined the lysozyme-catalyzed hydrolysis of the hexasaccharide of *N*-acetylglucosamine [$(GlcNAc)_6$] (Fig. 4 from Ref. 9) as a function of water content and showed that the reaction grows sharply at $w_1 > 0.15$.

The μ_2^E, $\overline{TS}_2^E$, and $\overline{H}_2^E$ values, and enzymatic activity, have different levels of dependency on hydration (Figs. 3 and 6 [this study] and Fig. 3 from Ref. 6). Enzymatic activity is suppressed within regimes 1 and 2 (Fig. 4 from Ref. 9).

The slow relaxation process[46] was activated at $w_1 > 0.15$ (Fig. 4 from Ref. 9). It has been suggested that the slow relaxation process might be correlated with the motions of the secondary protein structures. The dependence of the slow process on the water content is associated with the hydration dependency of the enzymatic activity of lysozyme and the rotation relaxation time of a probe located near the protein surface, in studies performed with electron paramagnetic resonance (EPR) spectroscopy[57] (Fig. 4 from Ref. 9). The slow relaxation process[46] is initiated, and the distribution of conformational states[55,57] is close to normal, only when the hydration of the protein surface attains a particular level. When the formation of the spanning hydrogen bond network of water is complete, the proteins then become catalytically active.

The most important observations are the correlations presented in Figs. 2, 3, 5, and 6. These correlations show that the contributions corresponding to the protein interior and the protein surface are coupled differently to the excess functions. The changes in the excess partial entropies and enthalpies corresponding to the protein interior are largely complete within regime 2. The changes in the excess partial quantities corresponding to the protein surface are complete within regime 3. Thus, it is probable that regime 3 corresponds to the completion of the formation of the spanning hydrogen-bonded network of water.

Regime 4 ($w_1 > 0.5$). At the highest water content, the proteins are in a flexible (elastic) state.[1] Excess partial quantities attain their fully hydrated values. The μ_2^E, $\overline{TS}_2^E$, and $\overline{H}_2^E$ values (Figs. 3 and 6 [this study] and Fig. 3 from Ref. 9) reach minimal values. At $w_1 < 0.5$, the $w_1\bar{Z}_1^E$ values are near zero (Figs. 4(a)–4(c)). Bulk water was observed in this region through proton NMR measurements.[45] The molecular motion of these water molecules is solely determined through the interaction characteristics of water molecules. Water addition has no significant effect on the excess thermodynamic functions. At $w_1 > 0.5$, the changes in the excess thermodynamic functions primarily reflect changes in the state of lysozyme.

The high water content region is the biologically important regime. Aggregation/association (protein-protein interactions) in the solutions at high water content is a topic of extensive research.[58–62] One of the possible reasons for this process is the disruption of the crucial hydration shell in the presence of low molecular additives (e.g., organic solvents and salts). One can approximate the thickness of the hydration shell for lysozyme at $w_1 > 0.5$. The water weight fraction of $w_1 = 0.5$ is equivalent to 1 g H_2O g^{-1} protein or 794 water molecules per lysozyme molecule. A monolayer water coverage was estimated from the proton NMR[45] (417 molecules) and sorption calorimetry[16] (420 molecules) measurements. This comparison shows that, at $w_1 = 0.5$, each lysozyme molecule is covered by two water layers. Assuming that the diameter of one water molecule is 0.285 nm,[63] the hydration shell of lysozyme may be estimated to be 0.57 nm. This value is consistent with the estimation presented in Ref. 4. The hydration shell of proteins was demonstrated to be 0.4–0.8 nm thick. On the basis of this value, we can estimate the minimal distance between the lysozyme surfaces (two water layers for each lysozyme molecule) at high water content; this value is 1.14 nm.

V. SUMMARY

A novel method was applied for simultaneously estimating the excess partial quantities of protein and water. This method is based on the analysis of the excess thermodynamic functions of mixing. Specifically, it facilitates the individual evaluation of protein and water excess partial quantities in the entire range of water content. In the present study, we applied this method to simultaneously monitor the excess partial Gibbs energies, enthalpies, and entropies of water and lysozyme.

The protein and water contributions to the excess functions were shown to markedly depend on the hydration level. At the lowest water content, changes in the excess functions solely reflect the addition of water. The protein's transition from a glassy (rigid) to a flexible (elastic) state is accompanied by significant changes in the excess partial quantities of the protein and water. This transition is reflected in the calculated quantities when the charged groups on the protein are covered, which occurs at a water weight fraction of 0.05 and at 25 °C.

Excess partial quantities reach fully hydrated values at a water weight fraction greater than 0.5, when the coverage of both polar and adjacent weakly interacting surface elements no longer changes appreciably upon additional hydration. At the highest water content, water addition has no significant effect on the excess functions. At $w_1 > 0.5$, changes in the excess functions primarily reflect changes in the state of the protein.

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