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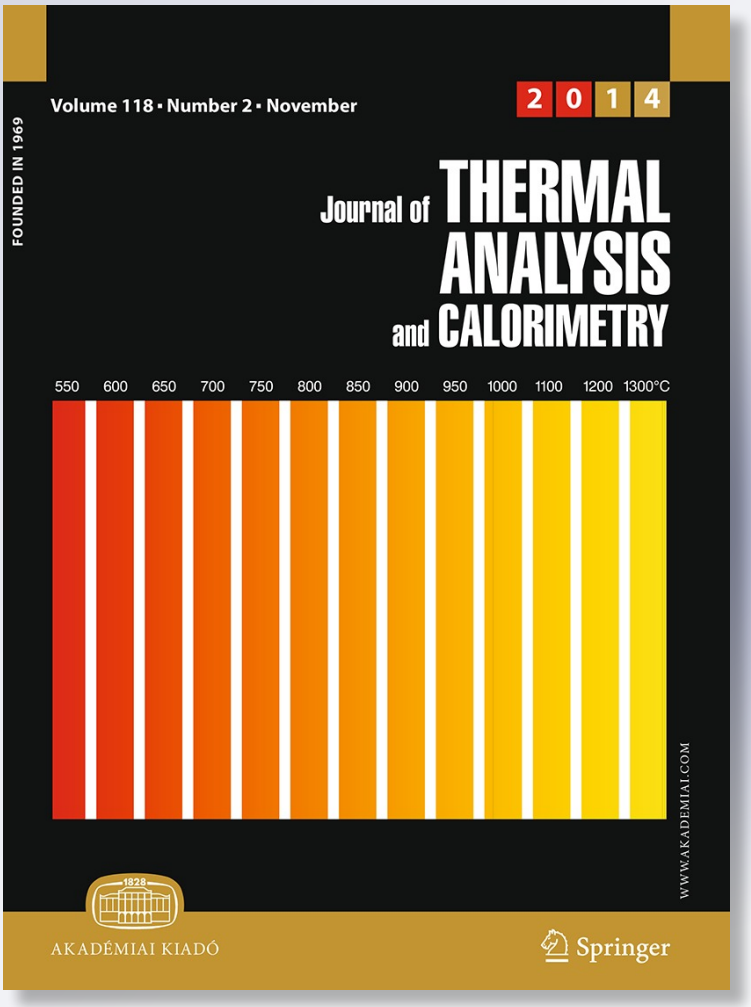

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Effect of the protein hydrophobicity and polarity

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Abstract This work is part of a systematic study undertaken to find the excess thermodynamic functions of binary protein–water systems. Isothermal calorimetry and water sorption measurements were applied to characterize the hydration dependencies of the excess thermodynamic functions. The advantages of our methodology are (i) we are able to simultaneously determine the excess partial quantities of water and proteins; (ii) these thermodynamic quantities can be determined in the entire range of water content. Here, in particular, the excess partial enthalpies of water and bovine pancreatic ribonuclease A (RNase A) have been determined. The excess partial enthalpies for RNase A are compared with the published data for several unrelated globular proteins (lysozyme, chymotrypsinogen A, serum albumin, lactoglobulin). These biomacromolecules represent a series of proteins in which the hydrophobicity of proteins is gradually changed in a wide range. It was found that the excess partial quantities for the studied proteins are determined by the hydration of the hydrophilic and hydrophobic protein groups. The more hydrophilic a protein, the more significant a hydrophilic hydration contribution is and vice versa. RNase A is the most hydrophilic protein under the study. This protein has the most significant hydrophilic hydration contribution. Lactoglobulin is the most hydrophobic protein under the study. This protein has the most significant hydrophobic hydration contribution.

Keywords Protein (biomacromolecule) hydration · Isothermal calorimetry · Excess functions · Enthalpy

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Introduction

Water binding (hydration or biological water) plays a crucial role in determining the structure, stability, dynamics, and functions of proteins [1–10]. There are essential differences between hydration and bulk water surrounding a protein [1–7, 11, 12]. Thus, understanding the hydration of proteins requires elucidating the effects of 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.

Thermochemical studies have traditionally been of great importance in ascertaining a better understanding of protein–water interactions. Herein, we present a review of the available literature concerning the hydration of proteins. Because our current research involves a thermochemical study of protein–water systems, this literature review will focus on studies concerning the corresponding enthalpy changes. More comprehensive reviews have been previously published in refs [1–4].

Isothermal calorimetry is one of the most effective methods for obtaining reliable thermochemical information on the interactions of proteins with water in various environments. Amberg measured the heats of water vapor sorption using bovine serum albumin within a water content range of 0–12 % (g g^{-1}) at 20 °C [13]. The enthalpies of solution of dried and hydrated ribonuclease A (RNase A) were measured using isothermal calorimetry at 25 °C [14]. The water content of RNase A 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. [15] calorimetrically measured the heat of water adsorption by lysozyme in the range of relative water vapor pressures from 0 to 0.895 and obtained both the sorption

isotherm and enthalpy of hydration of the enzyme within a water content range of 0–18 % (g g^{-1}) at 25 °C. 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 protein hydration over the entire range of water contents [17]. The interaction enthalpy depends significantly on the initial water content and hydration history. The most important observations of these calorimetric studies [13–17] can summarized as follows: The hydration enthalpies vary strongly with the hydration level. They are large at low hydration levels. As the water content increases, the hydration enthalpies approach the enthalpy of condensation of pure water.

The measured enthalpies [13–17] contain total information on 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 enthalpy (or other thermodynamic functions) of binary protein–water systems in the entire range of water contents has not been attempted [1–10, 13–16].

In our previous studies [11, 18], we proposed a novel method to investigate the protein–water interactions. This method is based on the analysis of the thermodynamic functions of mixing. It facilitates the individual evaluation of the protein and water partial quantities in the entire range of water content. The present study is aimed to understand what molecular parameters control the excess enthalpies of the binary protein–water systems.

To test the predictive ability of the proposed method, a system containing, as a model protein, bovine pancreatic ribonuclease A (RNase A) was studied in this work. The excess partial quantities for RNase A were compared with the published data for several unrelated globular proteins (lysozyme, chymotrypsinogen A, serum albumin, lactoglobulin) [11]. These biomacromolecules represent a series of proteins in which the hydrophobicity of proteins is gradually changed in a wide range. RNase A is one of the most hydrophilic proteins applied in biothermodynamic studies [19–21].

Ribonuclease A is a small monomeric enzyme of 124 amino acids, containing no non-enzyme components. Ribonuclease is an example of an $\alpha + \beta$ protein. RNase hydrolyzes single-stranded RNA through cleavage of the phosphodiester linkage at the 3′ side of pyrimidine nucleotides [19–21].

Methodology

Excess partial quantities

The thermodynamic properties of a real binary water–protein system can be expressed in terms of the excess functions, Z^{E} [22–24], 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:

$$Z^{E} = w_1 \overline{Z}^{E}_{1} + w_2 \overline{Z}^{E}_{2} \tag{3}$$

where $\overline{Z}^{E}_{1}$ is the excess partial function for component 1 (water), and $\overline{Z}^{E}_{2}$ is the excess partial function for component 2 (protein).

Excess partial enthalpies

The excess partial enthalpies of the protein and water were calculated as described previously [11, 18]. 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, $\Delta_{sol}H(\text{dried})$, is described in Eq. 4. The final state of the protein (Eqs. 4, 5) is a diluted solution in water at 25 °C and at atmospheric pressure:

$$\Delta_{sol}H(\text{dried}) = \left[\overline{H}^{sol}_{P} m_P\right] - \left[\overline{H}^{0}_{P} m_P\right] + \left[\overline{H}^{sol}_{w,protein} m^{sol}_{w}\right] - \left[\overline{H}_{w,liquid} m^{sol}_{w}\right] \tag{4}$$

where $\overline{H}^{0}_{P}$ 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; $\overline{H}^{sol}_{w,protein}$ is the partial enthalpy of water bound to the protein in the diluted aqueous solution; $\overline{H}^{sol}_{P}$ is the partial enthalpy of the protein in the solution; m_P is the amount of the protein; m^{h}_{w} is the amount of water

Table 1 Symbols used in section "Methodology"

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

bound to the protein; and $m_{\mathrm{w}}^{\mathrm{sol}}$ 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, $\Delta_{\mathrm{sol}}H$(hydrated), is described in Eq. 5:

$$\begin{aligned}\Delta_{\mathrm{sol}}H(\mathrm{hydrated}) = &\left[\overline{H}_{\mathrm{P}}^{\mathrm{sol}} m_{\mathrm{P}}\right] - \left[\overline{H}_{\mathrm{P}}^{\mathrm{h}} m_{\mathrm{P}}\right] + \left[\overline{H}_{\mathrm{w,protein}}^{\mathrm{sol}} m_{\mathrm{w}}^{\mathrm{sol}}\right] \\ &- \left[\overline{H}_{\mathrm{w,liquid}} m_{\mathrm{w}}^{\mathrm{sol}}\right] + \left[\overline{H}_{\mathrm{w,liquid}} m_{\mathrm{w}}^{\mathrm{h}}\right] \\ &- \left[\overline{H}_{\mathrm{w,protein}}^{\mathrm{h}} m_{\mathrm{w}}^{\mathrm{h}}\right] \end{aligned} \quad (5)$$

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

As shown in Eq. 6, the H^{E} value is calculated from the enthalpies of solution of the dried, $\Delta_{\mathrm{sol}}H$(dried), and hydrated, $\Delta_{\mathrm{sol}}H$(hydrated), protein in water, measured using isothermal calorimetry:

$$\begin{aligned}&\frac{\Delta_{\mathrm{sol}}H(\mathrm{dried}) - \Delta_{\mathrm{sol}}H(\mathrm{hydrated})}{m_{\mathrm{w}}^{\mathrm{h}} + m_{\mathrm{P}}} = \left[\overline{H}_{\mathrm{P}}^{\mathrm{h}} - \overline{H}_{\mathrm{P}}^{0}\right] \frac{m_{\mathrm{P}}}{m_{\mathrm{P}} + m_{\mathrm{w}}^{\mathrm{h}}} \\ &+ \left[\overline{H}_{\mathrm{w,protein}}^{\mathrm{h}} - \overline{H}_{\mathrm{w,liquid}}\right] \frac{m_{\mathrm{w}}^{\mathrm{h}}}{m_{\mathrm{P}} + m_{\mathrm{w}}^{\mathrm{h}}} \\ &= \left[\overline{H}_{\mathrm{P}}^{\mathrm{h}} - \overline{H}_{\mathrm{P}}^{0}\right] \mathrm{w}_2 + \left[\overline{H}_{\mathrm{w,protein}}^{\mathrm{h}} - \overline{H}_{\mathrm{w,liquid}}\right] \mathrm{w}_1 = H^{\mathrm{E}} \end{aligned} \quad (6)$$

The excess partial enthalpies of the protein and water are calculated using Eqs. 7 and 8:

$$\overline{Z}_1^{\mathrm{E}} = Z^{\mathrm{E}} - w_2 \left(\frac{\mathrm{d}Z^{\mathrm{E}}}{\mathrm{d}w_2}\right)_{\mathrm{T,P}} = \left[\overline{H}_{\mathrm{w,protein}} - \overline{H}_{\mathrm{w,liquid}}\right] = \overline{H}_1^{\mathrm{E}} \quad (7)$$

$$\overline{Z}_2^{\mathrm{E}} = Z^{E} - w_1 \left(\frac{\mathrm{d}Z^{\mathrm{E}}}{\mathrm{d}w_1}\right)_{\mathrm{T,P}} = \left[\overline{H}_{\mathrm{P}}^{\mathrm{h}} - \overline{H}_{\mathrm{P}}^{0}\right] = \overline{H}_2^{\mathrm{E}} \quad (8)$$

where $\overline{H}_1^{\mathrm{E}}$ (J g^{-1} water) and $\overline{H}_2^{\mathrm{E}}$ (J g^{-1} protein) 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. The symbols used in section "Methodology" are summarized in Table 1.

Experimental

Materials

Bovine pancreatic ribonuclease A of the highest commercially available purity was purchased from Sigma Chemical (St. Louis, MO, USA) and used without further purification. The purity of enzyme 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 mass of ribonuclease A was taken as 13,700 Da. The water was doubly distilled.

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 mass 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 [25]. This zero hydration level was described using $\overline{H}_P^0$ (Eqs. 4–6).

At the lowest and intermediate water mass 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 Ref [18]. 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 and KOH solutions were taken from [26, 27]. The following substances 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 mass was reached.

At the highest water mass fractions ($a_w > 0.98$), the water activity was adjusted by changing the unsaturated NaCl–water solutions at 25 °C. The a_w values of the NaCl–water solutions were taken from Ref. [28]. This technique was used for the protein–water systems for $w_1 > 0.6$.

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. 4, 5) were measured at 25 °C using a Setaram BT-2.15 calorimeter, according to a previously described procedure [29, 30]. 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 [31].

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 [32]. 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" [33]: $\Delta_{sol}H^{KCl/H_2O}$ (25 °C, m = 0.02775 mol kg^{-1}) = 17.47 ± 0.07 kJ mol^{-1}.

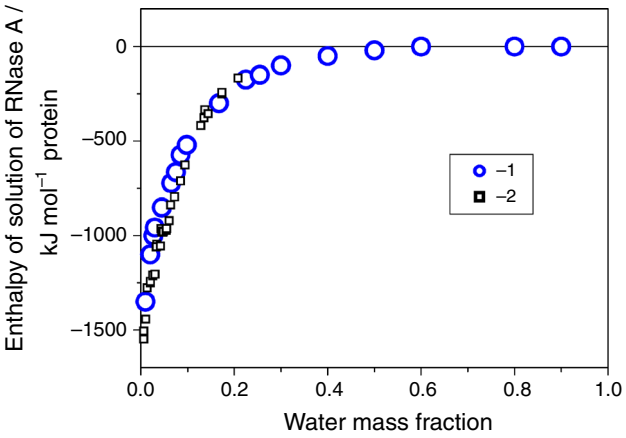

Fig. 1 The enthalpy of the solution of the hydrated RNase A, $\Delta_{sol}H$(hydrated) /m_P, in water as a function of the water mass fraction, w_1, at 25 °C. The standard errors of estimation of the $\Delta_{sol}H$(hydrated) /m_P values were 0.5–1.2 %. Each experiment was performed 3–4 times. *1* This study. *2* Adapted data form Ref. [14]

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 mass was reached. This zero hydration level was described using the $\overline{H}_P^0$ values (Eqs. 4–6).

At the lowest and intermediate water content, the protein preparations were prepared as described in section "Water sorption measurements." At the highest water content, the protein and water samples were mixed in the calorimetric cell at various water mass 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 mass was reached.

The hydrated protein samples were described using $\overline{H}_P^h$ and $\overline{H}_{w,protein}$ (Eqs. 5–8).

Results and discussion

Partial enthalpies of water and RNase A

Figure 1 presents the $\Delta_{sol}H$(hydrated) values (Eq. 5) as a function of the mass fraction of water. As shown in Fig. 1, the $\Delta_{sol}H$(hydrated) values are consistent with previously published results [14] indicating that the apparatus and the

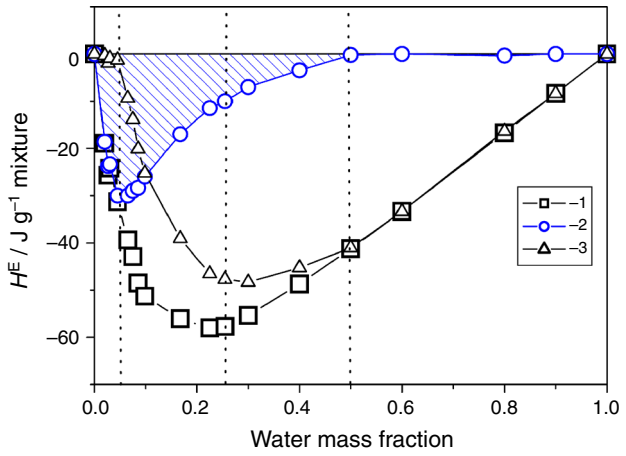

Fig. 2 Excess thermodynamic functions plotted versus the mass fraction of water, w_1, at 25 °C: *1* H^E; *2* $w_1\overline{H}_1^E$; *3* $w_2\overline{H}_2^E$. The standard errors of estimation of H^E were 0.8–2.2 %

experimental procedure are adequate to calculate the thermodynamic functions.

Figure 2 shows the dependency of the H^E values (excess functions of mixing per-unit-mass of the water–protein mixture) on the hydration level of RNase A. The H^E values were estimated using Eq. 6. Figure 3 shows the dependency of the $\overline{H}_1^E$ values on the hydration level of RNase A. The $\overline{H}_1^E$ values were calculated using Eq. 7. Figure 4 presents the $\overline{H}_2^E$ values as a function of the mass fraction of water. The $\overline{H}_2^E$ values were calculated using Eq. 8.

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 Fig. 2, the H^E values differ significantly from zero, indicating that the water–protein mixtures are non-ideal in the entire range of water content. Figure 2 presents the dependencies of the $w_i\overline{Z}_i^E$ ($w_1\overline{H}_1^E$ and $w_2\overline{H}_2^E$) functions plotted versus the water mass 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. Figure 2 demonstrates that the protein contribution ($w_2\overline{H}_2^E$) reaches maximal values at $w_1 \sim 0.25$. However, the $w_1\overline{H}_1^E$ function reaches maximal values at low water content ($w_1 \sim 0.05$).

The excess partial enthalpy curves presented in Figs. 3 and 4 can be divided into four parts.

At the lowest water content ($w_1 = 0$–0.05), the $\overline{H}_2^E$ values are close to zero and do not depend on the water content (Fig. 4). The proteins are in a glassy (rigid) state [1]. In the glassy state, the dehydration-induced conformational changes and restrictions on conformational transitions cause the protein to become frozen into a broad distribution of conformational states. Significant changes in the amide I region of the dehydrated proteins were observed using Fourier transform infrared spectroscopy [34–37]. Khurgin et al. [38] measured the chymotrypsin-catalyzed hydrolysis of the amide substrate *N*-succinyl-L-phenylalanine-p-nitroanilide at low and medium hydration levels. No enzymatic activity was observed at $w_1 < 0.05$ (Fig. 3 from Ref. [11]).

At $w_1 < 0.05$, the $w_2\overline{H}_2^E$ values are close to zero (Fig. 2). At $w_1 \sim 0.05$, the $w_1\overline{H}_1^E$ functions reach maximal values. These facts imply that, at the lowest water content, changes in the excess thermodynamic functions solely reflect water addition.

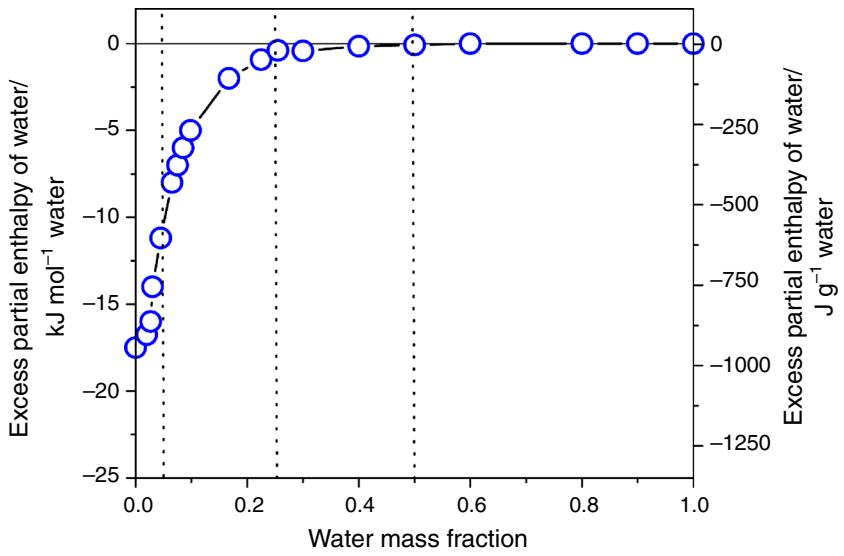

Fig. 3 Excess partial enthalpy of water, $\overline{H}_1^E$, as a function of the mass fraction of water, w_1, at 25 °C. The reference state is pure liquid water at 25 °C and atmospheric pressure

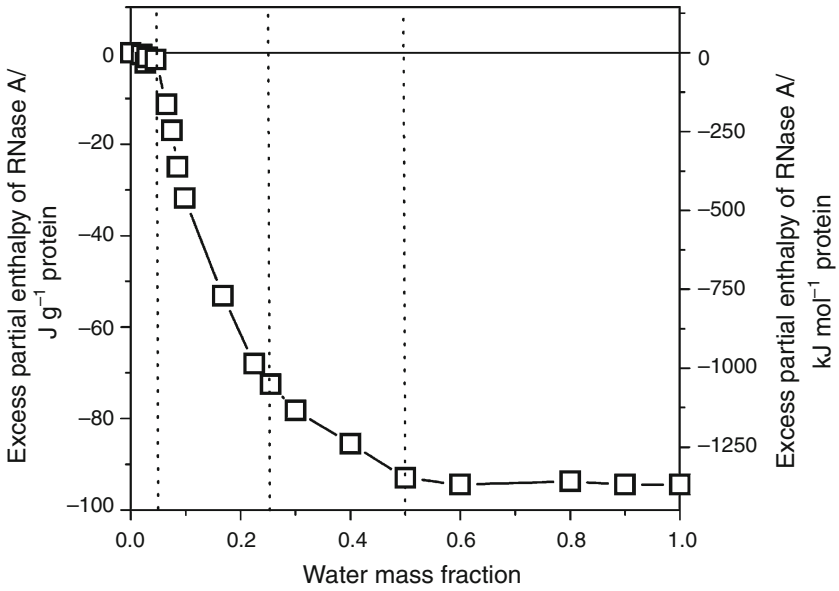

Fig. 4 Excess partial enthalpy of RNase A, $\overline{H}_2^{\mathrm{E}}$, as a function of the mass fraction of water, w_1, at 25 °C

Hutchens et al. [39] examined the heat capacities of insulin at $w_1 = 0$ and 0.038 and chymotrypsinogen A at $w_1 = 0$ and 0.096, from −263 to 37 °C. 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, 40]. At low water content, the $\overline{H}_1^{\mathrm{E}}$ values differ significantly from zero (Fig. 3). The $\overline{H}_1^{\mathrm{E}}$ values are highly negative. As concluded in Ref. [11], the $\overline{H}_1^{\mathrm{E}}$ ($w_1 = 0$) 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.

Part 2 ($w_1 = 0.05$–0.25) corresponds to the hydration of polar groups [1, 2, 40]. In this water content range, the $\overline{H}_1^{\mathrm{E}}$ values change sharply from highly negative to moderate values (Fig. 3). This sharp transition reflects the formation of a spanning hydrogen-bonded network of water at the protein surface [2, 5]. The formation of this network occurs via a quasi two-dimensional percolation transition of the hydration water at the enzyme surface [5].

The results obtained for biopolymers using several experimental methods have been summarized in Ref. [1]. The results suggest that proteins undergo a glasslike dynamic transition at water content of approximately 10 mass% at 25 °C. This water content falls within part 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, the apparent heat capacity of lysozyme, C_{p2}, determined from isothermal experiments using a drop calorimeter, increases from extremely low values to high values in this water content interval [41]. During the isothermal sorption of water, a glasslike transition results in a step on the excess partial enthalpy of RNase (Fig. 4). The $\overline{H}_2^{\mathrm{E}}$ values change sharply from extremely low values to highly negative values.

Part 3 ($w_1 = 0.25$–0.5) indicates the appearance of the "structured" water [40]. "Structured" water is composed of molecules that interact with hydrophobic surface patches on the protein-bridging between bound water molecules. Yang and Rupley [41] 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 onset of biological activity was observed in this region. Rupley and Careri [2] examined the lysozyme-catalyzed hydrolysis of the hexasaccharide of *N*-acetylglucosamine [$(\mathrm{GlcNAc})_6$] (Fig. 3 from Ref. [11]) as a function of water content and showed that the reaction grows sharply at $w_1 > 0.15$. Enzymatic activity was suppressed within regimes 1 and 2 (Fig. 3 from Ref. [11]).

At the highest water content ($w_1 > 0.5$), the proteins are in a flexible (elastic) state [1]. Excess partial quantities attain their fully hydrated values. The $\overline{H}_2^{\mathrm{E}}$ values (Fig. 4) reach minimal values. At $w_1 < 0.5$, the $w_1\overline{H}_1^{\mathrm{E}}$ values are near zero (Fig. 2). Bulk water was observed in this region through proton NMR measurements [40]. 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 RNase A.

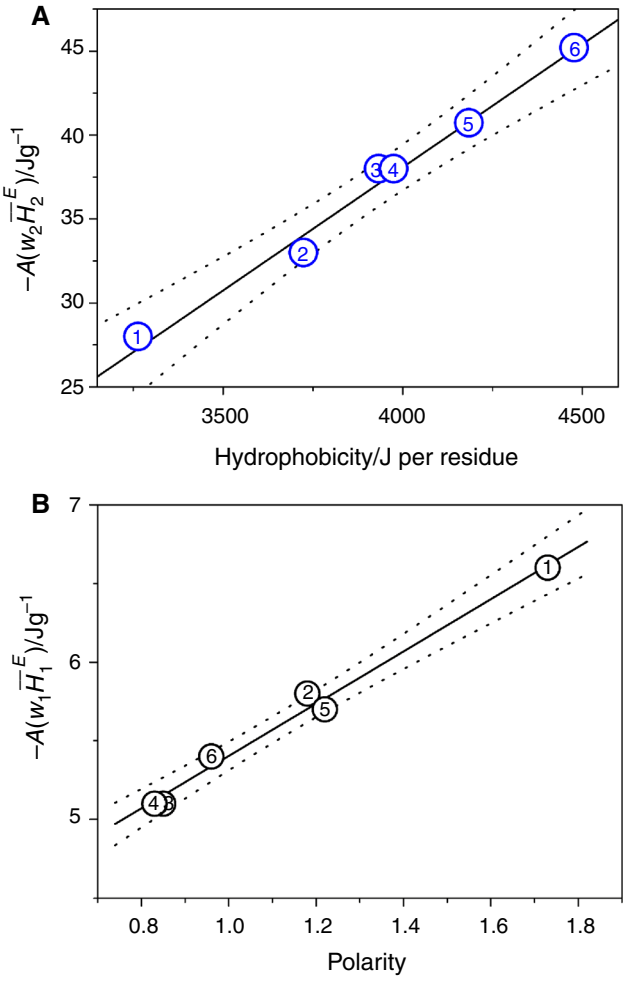

Fig. 5 *1* RNase A; *2* lysozyme; *3*; α-chymotrypsin; *4* chymotrypsinogen A; *5* serum albumin; *6* β-lactoglobulin. **a** Relationship between the $A(w_2\overline{H}_2^E)$ value and the hydrophobicity, H_ϕ, of proteins $-A(w_2\overline{H}_2^E) = -20.6\,(5.0) + 0.015\,(0.001) \times H_\phi$, where the number of the experimental points is $N = 6$, the standard error of estimation is $S_o = 1.2$, and the correlation coefficient is $R = 0.985$. The *dashed lines* show the 95 % confidence interval. **b** Relationship between the $A(w_1\overline{H}_1^E)$ value and Bigelow's polarity parameter, P, of proteins. $-A(w_1\overline{H}_1^E) = 3.7\,(0.1) + 1.7\,(0.1) \times P$, where the number of the experimental points is $N = 6$, the standard error of estimation is $S_o = 0.1$, and the correlation coefficient is $R = 0.993$. The *dashed lines* show the 95 % confidence interval

Effect of the protein hydrophobicity and polarity

To show the predictive ability of the proposed methodology, we related the obtained results to general properties of the proteins. Hydrophobicity and polarity were selected as possible common parameters. The hydrophobicity of the studied proteins has been estimated as the average hydrophobicity of the side chains of the constitutive amino acids, H_ϕ (J per residue). The calculated H_ϕ values for many proteins have been collected in Ref. [42].

We calculated the areas under the $w_1\overline{H}_1^E = f(w_1)$ and $w_2\overline{H}_2^E = f(w_1)$ curves for RNase A (this study), lysozyme, chymotrypsinogen A, α-chymotrypsin, β-lactoglobulin, and serum albumin [11]. These areas were defined as $A(w_1\overline{H}_1^E)$ and $A(w_2\overline{H}_2^E)$, respectively. The $A(w_1\overline{H}_1^E)$ area for RNase A is shown shaded in Fig. 2.

The $A(w_2\overline{H}_2^E)$ values are plotted as a function of H_ϕ in Fig. 5a. There is a clear correlation between the $A(w_2\overline{H}_2^E)$ values and H_ϕ. This correlation shows that the more hydrophobic a protein is the more significant the $A(w_2\overline{H}_2^E)$ value is.

A polarity parameter, P, was proposed by Bigelow [43]. It was defined as a volume ratio of polar amino acids to nonpolar amino acids. There is a good correlation between the $A(w_1\overline{H}_1^E)$ values and Bigelow's polarity parameter (Fig. 5b). This correlation means that the more hydrophilic a protein is the more significant the $A(w_1\overline{H}_1^E)$ value is.

As a rule, most hydrophobic groups of globular proteins are buried in the interior of the protein molecule. On the other hand, charged and polar residues are located preferentially on the surface. H_ϕ may be regarded as a parameter characteristic mostly of the hydrophobic interior of a protein. P reflects the degree of the polarity of the protein surface.

RNase A is the most hydrophilic protein under the study. As shown in Fig. 5b, this protein has the most significant hydrophilic hydration contribution, $A(w_1\overline{H}_1^E)$. Lactoglobulin is the most hydrophobic protein. As shown in Fig. 5a, this protein has the most significant hydrophobic hydration contribution, $A(w_2\overline{H}_2^E)$. These correlations show that the contributions corresponding to the hydration of the protein interior and the protein surface are coupled differently to the excess functions. The changes in the partial quantities corresponding to the protein interior are largely described by the $A(w_2\overline{H}_2^E)$ values. The changes in the partial quantities corresponding to the protein surface are described by the $A(w_1\overline{H}_1^E)$ values.

Conclusions

Isothermal calorimetry and water sorption measurements were applied to study the hydration of ribonuclease A. The hydration process was characterized by analyzing the excess functions of mixing. This method facilitates the individual evaluation of the protein and water partial quantities in the entire range of water content. The excess partial enthalpies are extremely sensitive to the changes in the state of water and the protein. It was shown that the

protein and water contributions to the excess functions markedly depend on the hydration level. At the lowest water content, changes of the excess functions solely reflect water addition. At the highest water content, water addition has no significant effect on the excess functions. At $w_1 > 0.5$, the changes of the excess functions primarily reflect changes in the state of the protein.

The excess partial enthalpies for RNase A are compared with the published data for several unrelated globular proteins (lysozyme, chymotrypsinogen A, serum albumin, lactoglobulin). It was found that the excess partial enthalpies for the studied proteins are determined by the hydration of hydrophilic and hydrophobic protein groups. The more hydrophilic a protein is, the more significant a hydrophilic hydration contribution is and vice versa. RNase A is the most hydrophilic protein under the study. This protein has the most significant hydrophilic hydration contribution. Lactoglobulin is the most hydrophobic protein under the study. This protein has the most significant hydrophobic hydration contribution.

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