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Hydration of Proteins: Excess Partial Enthalpies of Water and Proteins

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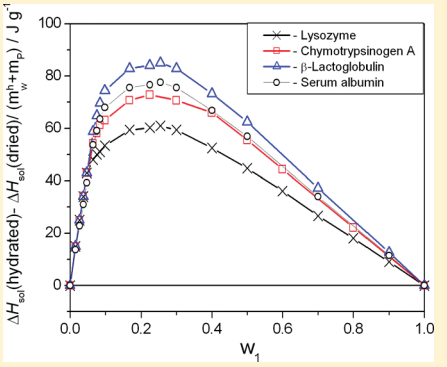
ABSTRACT: Isothermal batch calorimetry was applied to study the hydration of proteins. The hydration process was analyzed by the simultaneous monitoring of the excess partial enthalpies of water and the proteins in the entire range of water content. Four unrelated proteins (lysozyme, chymotrypsinogen A, human serum albumin, and β-lactoglobulin) were used as models. The excess partial quantities are very sensitive to the changes in the state of water and proteins. At the lowest water weight fractions (w_1), the changes of the excess thermochemical functions can mainly be attributed to water addition. A transition from the glassy to the flexible state of the proteins is accompanied by significant changes in the excess partial quantities of water and the proteins. This transition appears at a water weight fraction of 0.06 when charged groups of proteins are covered. Excess partial quantities reach their fully hydrated values at $w_1 > 0.5$ when coverage of both polar and weakly interacting surface elements is complete. At the highest water contents, water addition has no significant effect on the excess thermochemical quantities. At $w_1 > 0.5$, changes in the excess functions can solely be attributed to changes in the state of the proteins.



1. INTRODUCTION

The hydration of proteins is a subject of considerable interest to the chemist, physicist, and biologist. It is well-known that water bound to proteins (hydration or biological water) plays a key role in determining their stability, structure, dynamics, and functions.[1−6] On the other hand, the protein−water interactions mediate the properties of hydration water surrounding the protein.[1−7] Thermochemical studies have traditionally been of great importance in ascertaining a better understanding of protein−water interactions.[1,2,4] Therefore, a quantitative estimation of the protein and water contributions to the thermochemical functions of binary protein−water systems is of considerable biochemical importance and physicochemical interest.

Below a short review of the available studies on the hydration of proteins is given. Because our paper presents a calorimetric study of the protein hydration, a major focus of this section aims to discuss the thermochemical results. More comprehensive reviews have been given in refs 1−4.

Yang and Rupley[8] studied the apparent heat capacity of lysozyme as a function of water content. They identified four stages in the hydration process. Stage I (w_1 (water weight fraction) = 0−0.06) corresponds to hydration of charged groups. Stage II (w_1 = 0.06−0.2) corresponds to the saturation of the remaining polar sites probably associated with formation of clusters of water molecules. Stage III (w_1 = 0.2−0.28) represents the condensation 0of water over weakly interacting surface elements. Stage IV (w_1 = 0.28 to dilute solution) corresponds to the addition of water to the fully hydrated protein. The heat capacity measurements for lysozyme are in agreement with data obtained for other globular proteins (ovalbumin,[9] chymotrypsinogen, and insulin[10]).

This division into four steps is consistent with classifications derived from spectroscopic and dynamic measurements. For example, one of the most effective experimental approaches for studying the hydration of proteins is to evaluate changes in the motion of water molecules using nuclear magnetic resonance (NMR) measurements.[1,2,4,11−15] Fullerton et al.[12] identified four water fractions with different correlation times for water motions in the lysozyme−water systems, "superbound" (water molecules bonded to charged sites; $w_1 \approx 0-0.052$), "polar bound" (water molecules directly hydrogen bonded to polar sites on the protein macromolecule; $w_1 \approx 0.052-0.2$), "structured" (water molecules that are motionally perturbed by a protein but not bonded to it; $w_1 \approx 0.2-0.58$), and bulk. Lioutas et al.[16] performed similar experiments and also found three fractions of water with motional properties different from bulk water.

From the temperature dependence of the water sorption isotherms in the range of 17−57 °C, Hnojewyj and Reyerson calculated differential heats of water sorption.[17] Luscher-Mattli and Ruegg[18,19] calculated the enthalpy of water sorption by lysozyme and α-chymotrypsin. The hydration enthalpies were calculated from the temperature dependence of the water vapor pressure in the range of 25−40 °C. Bone studied the water sorption by lysozyme in the range of 1.5−19% (g/g).[20] Calculations were performed using the temperature dependence of the water vapor pressure in the range of 6−46 °C. 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 most important assumption of this method is that the hydration enthalpy does not depend on the temperature. However, in a strict manner, this is not correct because the heat

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capacities of both components of the hydration process (water and protein) depend significantly on the temperature.

Calorimetry is one of the most effective methods for obtaining reliable thermochemical information on the interactions of proteins with water in various environments. Amberg has measured the heats of water vapor sorption by bovine serum albumin in the water content range of 0−12% (g/g) at 20 °C.[21] The enthalpy of solution of the dried and hydrated ribonuclease A (RNase A) have been measured by isothermal calorimetry at 25 °C.[22] The water content of RNase A was varied from 0 to 26% (g/g). The enthalpy of solution was negative (exothermic). As the water content of RNase A increased, the enthalpy of solution approached zero. The enthalpy of transfer of water from the pure liquid to the protein was estimated from the enthalpies of solution. Smith et al. have calorimetrically measured the heats of water adsorption by lysozyme in the range of relative water vapor pressures from 0 to 0.895.[23] They obtained both the sorption isotherm and the enthalpy of hydration of the protein in the water content range of 0−18% (g/g) at 25 °C. Pineri et al.[24] have measured both the water sorption−desorption isotherms and the enthalpy of water sorption−desorption for collagen. The most important conclusion is the evidence of different states of water sorption corresponding 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.[25] Our research group has developed an experimental method for measuring the heat effects of hydration−dehydration of proteins over the whole range of thermodynamic water activities.[26−28] The interaction enthalpy was found to depend significantly on the initial water content and hydration history.

These enthalpies[17−28] (estimated from the temperature dependencies of water sorption and the calorimetrically measured heat effects) contain total information on the binary water−protein systems, including the corresponding conformational changes in the protein structure and glass transition. However, no attempt has been undertaken to simultaneously estimate the protein and water contributions to the enthalpy of binary protein−water systems in the entire range of water contents.

A similar situation has been observed for other thermodynamic functions. For example, the thermodynamic properties of BPTI (bovine pancreatic trypsin inhibitor) were studied by molecular dynamics simulation and normal-mode analysis.[29] The partial internal energies and entropies of BPTI and water were only computed for the dry and fully hydrated protein.[29] Apparent heat capacities of lysozyme[8] and BPTI[29] were calculated in the water content range from the dried enzyme to the fully hydrated limit. However, the apparent heat capacity of water was only estimated for dry and hydrated proteins.

A novel experimental method has recently been proposed for simultaneously studying the excess partial enthalpies of water and the protein.[7] This method is based on the analysis of the excess functions of mixing. In this work, we applied this method for the simultaneous monitoring of the excess partial thermochemical quantities of water and the proteins in the entire range of water contents. A major focus of our work of the protein hydration aims to find the excess partial enthalpies of water and the proteins and show how these quantities correlate with coverage of the proteins by the water molecules.

Four unrelated proteins (lysozyme, chymotrypsinogen A, human serum albumin (HSA), and β-lactoglobulin) were used as models. They are among the most studied and applied in protein physical chemistry.[30,31] Lysozyme is a small monomeric protein composed of 129 amino acid residues. Lysozyme is an example of an $\alpha+\beta$ protein. The physiological role of lysozyme is to hydrolyze polysaccharide chains.[30,31] HSA is the most abundant in blood serum and plays a number of important biological roles, including the divalent cation transport, fatty acid and drug complexation, and transport.[32] It consists of 585 amino acid residues. HSA is an example of a predominantly α-helix protein. α-Chymotrypsinogen A is a typical globular protein. It contains 9% helix and 34% β-sheet.[33] This protein consists of 246 amino acids residues.[34] β-Lactoglobulin is a small globular protein composed of 162 amino acid residues.[35] In aqueous media, it contains about 52% β-sheets and 8% α-helices. This milk protein is known to bind tightly one retinol molecule per monomer.[36]

2. METHODOLOGY

For the separate estimation of the protein and water contributions to the thermodynamic functions of binary water−protein mixtures, it is convenient to use the excess partial functions. The thermodynamic properties of a real binary system can be expressed in terms of the excess functions, Z^E, the difference between the observed thermodynamic function of mixing, Z^m, and the function for an ideal binary mixture, Z^m_{id} (eq 1)

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

Deviations of the excess functions from zero indicate the extent to which the studied binary system is nonideal due to strong specific interactions between components (first of all, hydrogen bonding and charge−charge interactions).

The Z^E values are composed of two components (eq 2)

$$Z^E = w_1\overline{Z}^E_1 + w_2\overline{Z}^E_2 \tag{2}$$

where $\overline{Z}^E_1$ is the excess partial function for component 1 (water), $\overline{Z}^E_2$ is the excess partial function for component 2 (protein), and w_1 and w_2 are the mass fractions of water and enzyme, respectively.

The correct analysis of the thermochemical data requires the definition of the system under study. The first consideration is the case when:

(1) Initially, the solid protein phase does not contain water.

(2) The transfer of water from the gas phase to the protein phase occurs at 25 °C and atmospheric pressure. Then, the enthalpy change corresponding to the mixing of the dried protein with water is given by eq 3

$$\begin{aligned}\Delta H_{\mathrm{Solid-Gas}}(\mathrm{dried}) = {} & [\overline{H}_w m_w]_{\mathrm{finalsolid}} + [\overline{H}_w m_w]_{\mathrm{finalgas}} \\ & + [\overline{H}^h_P m_P]_{\mathrm{finalsolid}} - [\overline{H}_w m_w]_{\mathrm{initialgas}} \\ & - [\overline{H}^0_P m_P]_{\mathrm{initialsolid}}\end{aligned} \tag{3}$$

where $\overline{H}_P$ and $\overline{H}_w$ are the partial enthalpies of the protein and water, respectively, and where m_P and m_w are mass amounts of the protein and water, respectively. Phases (gas or solid) and states (final or initial) are specified by subscripts. The amount of water, m^{tr}_w, transferred from the gas phase to the protein phase is defined as in eq 4

$$\begin{aligned}m^{tr}_w &= [m_{w,\,\mathrm{final}} - m_{w,\,\mathrm{initial}}]_{\mathrm{solid}} \\ &= [m_{w,\,\mathrm{initial}} - m_{w,\,\mathrm{final}}]_{\mathrm{gas}} = [m_{w,\,\mathrm{final}}]_{\mathrm{solid}}\end{aligned} \tag{4}$$

The partial enthalpy of water in the gas phase is not changed during the interaction with the protein. Therefore, eq 3 can be

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transformed into eq 5

$$\begin{aligned}\Delta H_{\mathrm{Solid-Gas}}(\mathrm{dried}) &= [\bar{H}_{\mathrm{P,\,final}} - \bar{H}_{\mathrm{P,\,initial}}]_{\mathrm{solid}} \times m_{\mathrm{P}}[\bar{H}_{\mathrm{w}} m_{\mathrm{w}}]_{\mathrm{solid}} \\ &\quad + \bar{H}_{\mathrm{w,\,gas}}[m_{\mathrm{w,\,finalgas}} - m_{\mathrm{w,\,initialgas}}] \\ &= [\bar{H}^{\mathrm{h}}_{\mathrm{P,\,final}} - \bar{H}^{0}_{\mathrm{P,\,initial}}]_{\mathrm{solid}} \times m_{\mathrm{P}} \\ &\quad + [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,gas}}] m^{\mathrm{tr}}_{\mathrm{w}} \end{aligned} \quad (5)$$

The hydration of proteins can be characterized by two different methods.

2.1. Method 1. This is a typical way.[17−20] The $\Delta H_{\mathrm{Solid-Gas}}$(dried) value is related to the amount of water bound to the protein, $m^{\mathrm{h}}_{\mathrm{w}}$, (eq 6)

$$\Delta H^{\mathrm{H_2O/gas}}_{\mathrm{hydr}} = [\bar{H}^{\mathrm{h}}_{\mathrm{P}} - \bar{H}^{0}_{\mathrm{P}}]_{\mathrm{solid}} \frac{m_{\mathrm{E}}}{m^{\mathrm{h}}_{\mathrm{w}}} + [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,gas}}] \quad (6)$$

The $\Delta H^{\mathrm{H_2O/gas}}_{\mathrm{hydr}}$ value is called sometimes the enthalpy of protein hydration. As can be concluded from eq 6, Method 1 does not allow for studying separately the protein and water enthalpic contributions to the calorimetrically measured heat effects. According to eq 6, the $\Delta H^{\mathrm{H_2O/gas}}_{\mathrm{hydr}}$ value contains two components. The first component, $([\bar{H}^{\mathrm{h}}_{\mathrm{P}} - \bar{H}^{0}_{\mathrm{P}}]_{\mathrm{solid}})(m_{\mathrm{P}}/m^{\mathrm{h}}_{\mathrm{w}})$, is due to changes in the protein state during hydration (including conformational rearrangements and glass transition). The second component, $[\bar{H}_{\mathrm{w,solid}} - \bar{H}_{\mathrm{w,gas}}]$, is due to the difference between the water state in the gas phase and the bound state.

2.2. Method 2. The $\Delta H_{\mathrm{Solid-Gas}}$(dried) value observed during the addition of water to protein can be considered as an excess quantity of the mixing of water with protein (Z^{E}, eqs 1 and 2). From this quantity, the excess partial enthalpies can be calculated using eqs 7 and 8

$$\bar{Z}^{\mathrm{E}}_{1} = Z^{\mathrm{E}} - w_2 \left(\frac{\partial Z^{\mathrm{E}}}{\partial w_2}\right)_{T,P} = [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,gas}}] = \bar{H}^{\mathrm{E}}_{1} \quad (7)$$

$$\bar{Z}^{\mathrm{E}}_{2} = Z^{\mathrm{E}} - w_1 \left(\frac{\partial Z^{\mathrm{E}}}{\partial w_1}\right)_{T,P} = [\bar{H}^{\mathrm{h}}_{\mathrm{P}} - \bar{H}^{0}_{\mathrm{P}}]_{\mathrm{solid}} = \bar{H}^{\mathrm{E}}_{2} \quad (8)$$

where $\bar{H}^{\mathrm{E}}_{1}$ and $\bar{H}^{\mathrm{E}}_{2}$ are the excess partial enthalpies of water and protein, respectively and w_1 and w_2 are the mass fractions of water and protein, respectively. As can be concluded from eqs 7 and 8, Method 2 allows for studying separately the protein and water contributions to the calorimetrically measured heat effects.

Equation 5 can be transformed into an expression useful for experimental applications. When the transfer of water from the liquid phase to the protein phase occurs, eq 5 can be transformed into eq 9

$$\begin{aligned}\Delta H_{\mathrm{Solid-Liquid}}(\mathrm{dried}) &= [\bar{H}^{\mathrm{h}}_{\mathrm{P,\,final}} - \bar{H}^{0}_{\mathrm{P,\,initial}}]_{\mathrm{solid}} m_{\mathrm{P}} \\ &\quad + [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,liquid}}] m^{\mathrm{tr}}_{\mathrm{w}} \end{aligned} \quad (9)$$

The enthalpy change corresponding to introducing some amount of protein in pure liquid water (the solution enthalpy, $\Delta_{\mathrm{sol}}H$) can be defined by eqs 10 and 11. Let us consider two variants. In both cases, the final state of the protein is a diluted solution in water at 25 °C and atmospheric pressure.

2.3. Variant 1. Initially, the solid protein does not contain water.

$$\begin{aligned}\Delta_{\mathrm{sol}}H(\mathrm{dried}) &= [\bar{H}^{\mathrm{b}}_{\mathrm{P}} m_{\mathrm{P}}]_{\mathrm{finalliquid}} + [\bar{H}^{\mathrm{b}}_{\mathrm{w}} m^{\mathrm{b}}_{\mathrm{w}}]_{\mathrm{finalprotein}} \\ &\quad - [\bar{H}_{\mathrm{w}} m^{\mathrm{b}}_{\mathrm{w}}]_{\mathrm{initialliquid}} - [\bar{H}^{0}_{\mathrm{P}} m_{\mathrm{P}}]_{\mathrm{initialprotein}} \end{aligned} \quad (10)$$

where $\bar{H}^{\mathrm{b}}_{\mathrm{w}}$ is the partial enthalpy of water bound to the protein in the solution, $\bar{H}^{\mathrm{b}}_{\mathrm{P}}$ is the partial enthalpy of the protein in the solution and $m^{\mathrm{b}}_{\mathrm{w}}$ is the amount of water bound to the protein in the solution.

2.4. Variant 2. The initial protein phase may contain water.

$$\begin{aligned}\Delta_{\mathrm{sol}}H(\mathrm{hydrated}) &= [\bar{H}_{\mathrm{w}} m^{\mathrm{h}}_{\mathrm{w}}]_{\mathrm{finalliquid}} + [\bar{H}^{\mathrm{b}}_{\mathrm{P}} m_{\mathrm{P}}]_{\mathrm{finalliquid}} \\ &\quad + [\bar{H}^{\mathrm{b}}_{\mathrm{w}} m^{\mathrm{b}}_{\mathrm{w}}]_{\mathrm{finalprotein}} - [\bar{H}_{\mathrm{w}} m^{\mathrm{b}}_{\mathrm{w}}]_{\mathrm{initialliquid}} \\ &\quad - [\bar{H}^{\mathrm{h}}_{\mathrm{P}} m_{\mathrm{P}} + \bar{H}_{\mathrm{w}} m^{\mathrm{h}}_{\mathrm{w}}]_{\mathrm{initialprotein}} \end{aligned} \quad (11)$$

where $\bar{H}_{\mathrm{P}}$ and $\bar{H}_{\mathrm{w}}$ are the partial enthalpies of the protein and water, $m^{\mathrm{h}}_{\mathrm{w}}$ is the amount of water bound to initial solid protein, and $m^{\mathrm{b}}_{\mathrm{w}}$ is the amount of water bound to the protein in the solution.

The amount of water transferred from the liquid phase to the protein phase is defined by eq 12 when the initial protein does not contain water

$$m^{\mathrm{tr}}_{\mathrm{w}} = m^{\mathrm{b}}_{\mathrm{w}} \quad (12)$$

The amount of water transferred from the liquid phase to the protein phase is defined by eq 13 when the initial protein contains water

$$m^{\mathrm{tr}}_{\mathrm{w}} = [m^{\mathrm{b}}_{\mathrm{w,\,final,\,protein}} - m^{\mathrm{h}}_{\mathrm{w,\,initial,\,protein}}] \quad (13)$$

The difference between the solution enthalpies for the dried and hydrated proteins is defined by eq 14. It is expected that the partial enthalpy of water in the liquid phase does not change significantly during the formation of the diluted protein solution.

$$\begin{aligned}&\frac{\Delta_{\mathrm{sol}}H(\mathrm{dried}) - \Delta_{\mathrm{sol}}H(\mathrm{hydrated})}{m^{\mathrm{h}}_{\mathrm{w}}} \\ &= [\bar{H}^{\mathrm{h}}_{\mathrm{P}} - \bar{H}^{0}_{\mathrm{P}}]_{\mathrm{solid}} \frac{m_{\mathrm{P}}}{m^{\mathrm{h}}_{\mathrm{w}}} + [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,liquid}}] \\ &= \frac{\Delta H_{\mathrm{Solid-Gas}}(\mathrm{dried})}{m^{\mathrm{h}}_{\mathrm{w}}} + [\bar{H}_{\mathrm{w,\,gas}} - \bar{H}_{\mathrm{w,\,liquid}}] \\ &= \frac{\Delta H_{\mathrm{Solid-Gas}}(\mathrm{dried})}{m^{\mathrm{h}}_{\mathrm{w}}} + \Delta H^{\mathrm{H_2O}}_{\mathrm{vap}} \end{aligned} \quad (14)$$

where $\Delta H^{\mathrm{H_2O}}_{\mathrm{vap}}$ is the enthalpy of vaporization of water (43.7 kJ mol^{-1}).[44]

As can be concluded from eq 14, the Z^{E} value (eqs 1, 2, 7, and 8) can be calculated from the enthalpies of solution of the dried, $\Delta_{\mathrm{sol}}H$(dried), and hydrated, $\Delta_{\mathrm{sol}}H$(hydrated), proteins in water measured by isothermal calorimetry.

Equation 15 is similar to eq 2. It allows for calculating the excess partial quantities from the calorimetrically measured heat effects.

$$\begin{aligned}&\frac{\Delta_{\mathrm{sol}}H(\mathrm{dried}) - \Delta_{\mathrm{sol}}H(\mathrm{hydrated})}{m^{\mathrm{h}}_{\mathrm{w}} + m_{\mathrm{P}}} \\ &= [\bar{H}^{\mathrm{h}}_{\mathrm{P}} - \bar{H}^{0}_{\mathrm{P}}]_{\mathrm{solid}} \frac{m_{\mathrm{P}}}{m_{\mathrm{P}} + m^{\mathrm{h}}_{\mathrm{w}}} \\ &\quad + [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,liquid}}] \frac{m^{\mathrm{h}}_{\mathrm{w}}}{m_{\mathrm{P}} + m^{\mathrm{h}}_{\mathrm{w}}} \\ &= [\bar{H}^{\mathrm{h}}_{\mathrm{P}} - \bar{H}^{0}_{\mathrm{P}}]_{\mathrm{solid}} w_2 + [\bar{H}_{\mathrm{w,\,solid}} - \bar{H}_{\mathrm{w,\,liquid}}] w_1 \end{aligned} \quad (15)$$

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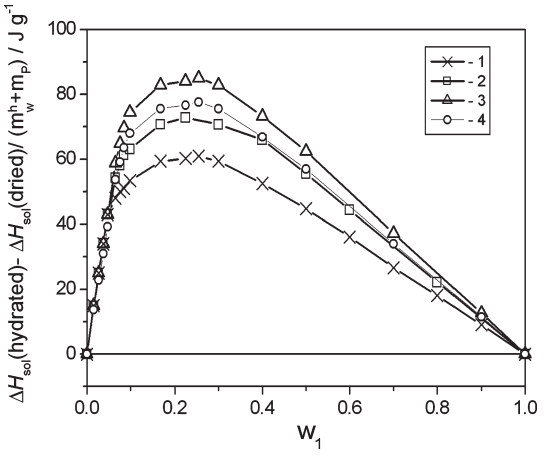

Figure 1. The $(\Delta_{sol}H(\text{hydrated}) - \Delta_{sol}H(\text{dried}))/(m_w^h + m_P)$ values as functions of the weight fraction of water, w_1, at 25 °C: (1) lysozyme; (2) chymotrypsinogen A; (3) HSA; and (4) β-lactoglobulin. Standard errors of estimation of the $(\Delta_{sol}H(\text{hydrated}) - \Delta_{sol}H(\text{dried}))/(m_w^h + m_P)$ values were 1−1.5 J g^{-1} mixture. Each experiment was performed 3−4 times.

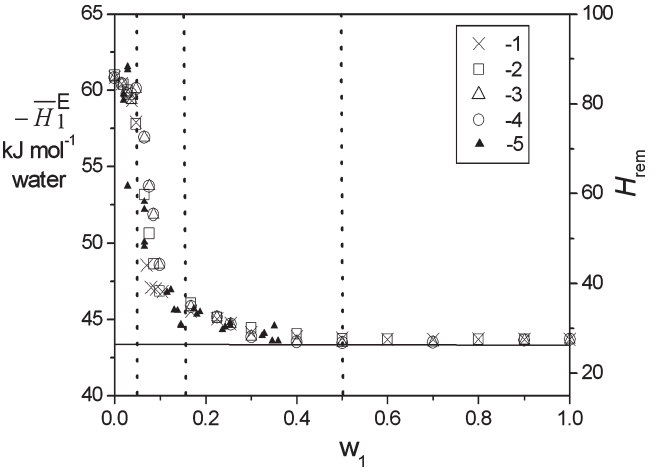

Figure 2. Excess partial enthalpies of water, $\overline{H}_1^E$, as functions of the weight fraction of water, w_1, at 25 °C: (1) lysozyme; (2) chymotrypsinogen a; (3) HSA; and (4) β-lactoglobulin. Excess partial enthalpies of water were corrected for the enthalpy of condensation of water at 25 °C. (5) Hydration dependence of amide hydrogen exchange in lysozyme powder at pH 5 and 25 °C. H_{rem} represents the number of hydrogens remaining unexchanged. Adapted data from ref 51.

3. EXPERIMENTAL SECTION

3.1. Materials. Hen egg-white lysozyme, HSA, bovine α-chymotrypsinogen A, and bovine β-lactoglobulin of the highest commercially available purity were purchased from Sigma Chemical (St. Louis, MO) and used without further purification. The purity of protein samples was proven by electrophoresis to be 95−98%. The molecular weights of proteins were taken as 14 300 Da (lysozyme), 66 000 Da (HSA), 18 200 Da (β-lactoglobulin), and 25 700 Da (chymotrypsinogen A). Water used was doubly distilled.

3.2. Calorimetry. Calorimetric experiments were conducted following the procedures described in detail elsewhere.[37−39] The enthalpy changes on the immersion of the dried proteins into pure liquid water were measured at 25 °C with a Setaram BT-2.15 calorimeter according to the described procedure.[38,39] Typically, the sample of 8−10 mg of protein was prepared with 4.0 mL of water in the calorimetric cell. The typical time of the calorimetric experiment was about 40 min. A typical heat evolution curve recorded upon dissolution of solid proteins in pure liquid water is given in refs 38 and 39.

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 according to the recommendation given in ref 40. The measured value of the solution enthalpy, $\Delta_{soln}H^{KCl/H_2O}$(298.15 K, m = 0.0277 mol kg^{-1}) = 17.43 ± 0.08 kJ mol^{-1}, is in a good agreement with the data recommended by the ICTAC working group "thermochemistry",[41] $\Delta_{soln}H^{KCl/H_2O}$(298.15 K, m = 0.02775 mol kg^{-1}) = 17.47 ± 0.07 kJ mol^{-1}.

The dried protein preparation (zero hydration level) was obtained by drying under vacuum using a microthermoanalyzer Setaram MGDTD-17S at 25 °C and 0.1 Pa until the constant sample weight was reached. The water content of the dried proteins was estimated to be 0.003 ± 0.002 g/g by the Karl Fischer titration method according to the recommendations.[39]

The initially dehydrated protein samples were exposed to pure water vapor. Water vapor was consecutively flowed through the thermostatted glass tube with drying agent (P_2O_5) and the thermostatted saturator filled with saturated salt solution and then through the cell containing the protein sample. The sorption equilibrium was reached after 30−60 min. Schematic representation of the experimental setup is given in refs 42 and 43. The water activity (a_w) in the vapor phase was adjusted by changing the saturated salt solution in the saturator. Water activities over saturated salt solutions were taken from refs 44 and 45. 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), and KNO_3 (a_w = 0.94). Salts for the conditioning of the samples were of analytical pure grade. The conditioned samples were equilibrated in the measuring cell at 25 °C before the experiment.

At the highest a_w values, the protein and water samples were mixed in the calorimetric cell at various water weight fractions and 25 °C. The masses of protein samples used in the equilibration were in the range of 8−10 mg. 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 the constant sample weight was reached.

4. RESULTS AND DISCUSSION

4.1. Excess Partial Enthalpies of Water and Proteins. Figure 1 shows how the $[\Delta_{sol}H(\text{hydrated}) - \Delta_{sol}H(\text{dried})]/(m_w^h + m_P)$ values (excess function of mixing per unit mass of the mixture) depend on the hydration level of the proteins. 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. Nonideal mixtures are composed of particles for which W−W, P−P, and W−P interactions are all different. The $(\Delta_{sol}H(\text{hydrated}) - \Delta_{sol}H(\text{dried}))/(m_w^h + m_P)$ values differ essentially from zero, indicating that the W−P mixtures are nonideal in the entire range of water contents. The most significant deviations were observed at $w_1 \approx 0.2-0.3$. This result means that at $w_1 \approx 0.2-0.3$, the water−protein interactions are more favorable (attractive) than the W−W and

P−P interactions in terms of enthalpy. At high and low water contents, the situation is similar. However, the dominance of the W−P interactions is less than that at $w_1 \approx 0.2-0.3$.

Figures 2 and 3A−C present the excess partial enthalpy of water, $\overline{H}_1^E$, and the excess partial enthalpy of the proteins, $\overline{H}_2^E$, as functions of the weight fraction of water. These thermochemical quantities were calculated using eqs 7, 8, and 15. The excess partial enthalpy curves presented in Figures 2 and 3 can be divided into four parts.

4.1.1. Regime 1. $w_1 = 0-0.06$ ($h \approx 0-0.07$ g/g). The main features of regime 1 can be described as follows. At the lowest water contents, the proteins are in a glassy (rigid) state.[1,25] 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. No biological activity was observed at the lowest water contents (Figures 3A and 4). At low water contents, the $\overline{H}_2^E$ values are close to zero and do not depend noticeably on the water content (Figure 3A−C). The fact that the proteins are in the glassy state explains this feature of regime 1. The $\overline{H}_2^E$ value is close to zero due to the fact that all of the protein molecules came into contact mainly with the same protein molecules during this range of water contents.

At the lowest water contents, the $\overline{H}_1^E$ values are highly exothermic (negative) (Figure 2). The $\overline{H}_1^E$ values do not depend noticeably on the water content and equal approximately −61.0 kJ mol^{-1} of water. The $\overline{H}_1^E - w_1$ curves do not depend noticeably on the nature of the protein.

Hutchens et al.[10] studied the heat capacities of insulin at $w_1 = 0$ and 0.038 and of 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 hydration of charged groups.[1,2,12] Certain conclusions regarding the nature of the water sorption sites of the dehydrated proteins can be made by analyzing the enthalpies of solvation of water in various organic solvents. The $\overline{H}_1^E$ values were compared with the enthalpies of solvation of water in organic solvents at infinite dilution, $\Delta_{solv}H^{H_2O/S}$, in Table 1. The enthalpy of solvation, $\Delta_{solv}H^{A/S}$, is the enthalpy of isothermal transfer of solute A (H_2O) from the ideal gas state to an infinitively diluted solution in solvent S (organic solvent) at 25 °C and 0.1 MPa. The solution enthalpy, $\Delta_{sol}H^{A/S}$, is the enthalpy of transfer of solute A from its reference state to an infinitively diluted solution in solvent S at 25 °C and 0.1 MPa. The $\Delta_{solv}H^{H_2O/S}$ and $\Delta_{sol}H^{A/S}$ values in organic solvents were calculated using eqs 16−18

$$\Delta_{solv}H^{H_2O/S} = \Delta_{sol}H^{H_2O/S} - \Delta H_{vap}^{H_2O} \quad (16)$$

where $\Delta_{sol}H^{H_2O/S}$ is the enthalpy of a solution of water in organic solvent, in kJ mol^{-1}; $\Delta H_{vap}^{H_2O}$ is the enthalpy of vaporization of water, 43.7 kJ mol^{-1}.

$$H^E - x_2\left(\frac{\partial H^E}{\partial x_2}\right)_{T,P} = \overline{H}_1^E \quad (17)$$

where H^E is the excess enthalpy of mixing, $\overline{H}_1^E$ is the excess partial enthalpy of water in organic solvent, and x_2 is the mole fraction of organic solvent.

$$\Delta_{sol}H^{H_2O/S} = \overline{H}_1^E - \overline{H}_1^0 \quad (18)$$

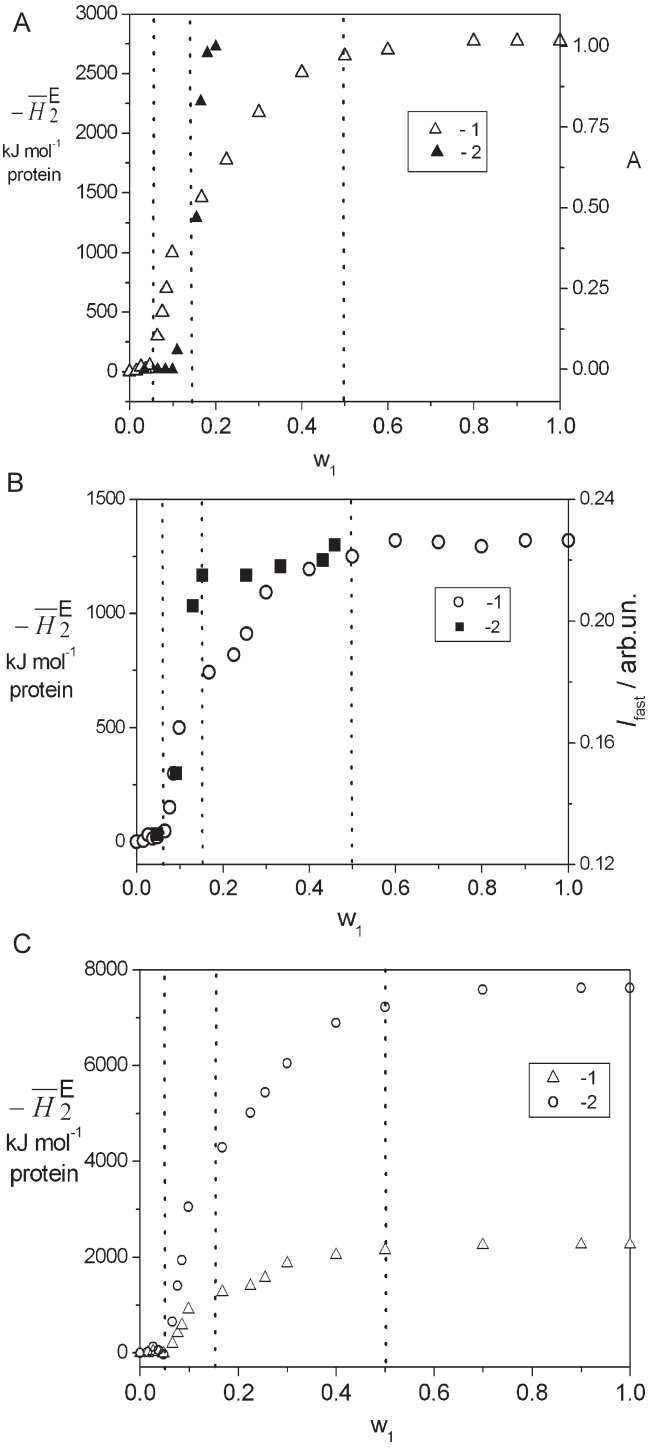

Figure 3. (A) (1) Excess partial enthalpy of chymotrypsinogen A, $\overline{H}_2^E$, as a function of the weight fraction of water, w_1, at 25 °C. (2) Solid-state solvent-free hydrolysis of *N*-succinyl-L-phenylalanine-*p*-nitroanilide. The ordinate, $A = D_{416}/D_{357}$, is a measure of the nitroaniline product. Modified data from ref 48. Water contents of α-chymotrypsin were taken from ref 42 with permission of Elsevier. (B) (1) Excess partial enthalpy of lysozyme, $\overline{H}_2^E$, 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 50 with permission of Elsevier. (C) (1) Excess partial enthalpy of β-lactoglobulin, $\overline{H}_2^E$, as a function of the weight fraction of water, w_1, at 25 °C. (2) Excess partial enthalpy of HSA, $\overline{H}_2^E$, as a function of the weight fraction of water, w_1, at 25 °C.

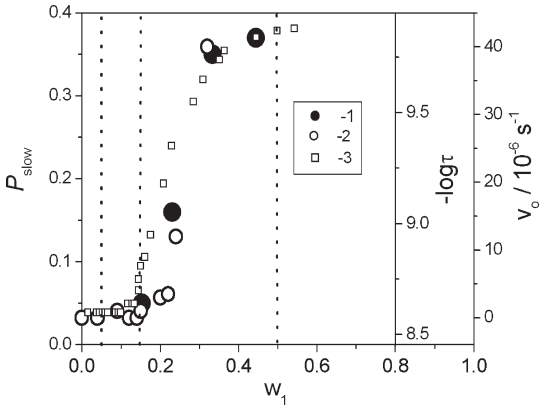

Figure 4. (1) Hydration dependence of the mobile fraction of hydrogen atoms involved in the slow relaxation process, P_{slow}. Adapted data from ref 50 with permission of Elsevier. (2) Enzymatic activity of lysozyme as a function of water content at pH 8 and 25 °C. Powder samples were the 1:1 $(GlcNAc)_6$—lysozyme complex. The reaction rate, v_o, was determined by product analysis. Adapted data from ref 49 with permission of Elsevier. (3) Rotational relaxation time of an EPR probe, $-\log\tau$. Adapted data from ref 49.

Table 1. Enthalpies of Solvation of Water in Organic Solvents at Infinite Dilution and 25 °C

solvent	$\Delta_{solv}H^{H_2O/S}$, kJ mol^{-1}
diethylamine	−58.1 (18 °C)[46]
acetic acid	−40.9[47]
N,*N*-dimethylformamide	−47.4[47]
ethanol	−45.7[47]

where $\overline{H}_1^0$ is the partial enthalpy of water in the reference state (pure liquid water at 25 °C and 0.1 MPa).

Diethylamine, acetic acid, ethanol and *N*,*N*-dimethylformamide (DMFA) were used as analogues of various protein groups. Acetic acid was used as an analogue of the amino acids containing the side carboxylic groups. Diethylamine was used as an analogue of the amino acids containing the side amino groups. DMFA was used as a low molecular analogue of the polypeptide backbone. Ethanol was used as an analogue of the amino acids containing the side OH groups.

As can be concluded from Table 1, the $\overline{H}_1^E$ value is close to that observed for diethylamine, which has the most exothermic excess partial enthalpy of water from the solvents under study (−58.1 kJ mol^{-1}). This result suggests that at the lowest w_1 values, the interaction with the amino groups may be a dominant factor controlling the state of water molecules.

4.1.2. Regime 2 (w_1 = 0.06−0.15). The main features of regime 2 are the following. The results obtained for biopolymers by several experimental methods have been summarized in ref 1. It was concluded that proteins undergo a glasslike dynamic transition at a water content of about 10 wt % at 25 °C. This water content is within regime 2 in this work. The transition from the glassy (rigid) to the flexible (elastic) state is accompanied by significant changes in the properties of proteins.[1] For example, the apparent heat capacity of lysozyme, ϕC_{p2}, determined from isothermal experiments using a drop calorimeter increases from very low values to high values in this water content interval.[8] During isothermal sorption of water, a glasslike transition results in a step on the excess partial enthalpy of the proteins (Figure 3A−C). The $\overline{H}_2^E$ values change sharply from very low values to highly exothermic ones.

Regime 2 corresponds to hydration of polar groups.[1,2,12] In the water content range w_1 = 0.06−0.15, the $\overline{H}_1^E$ values change sharply from highly negative (∼−61 kJ mol^{-1}) to moderate (∼−46 kJ mol^{-1}) values (Figure 2). This sharp transition was attributed to the formation of a spanning hydrogen-bonded network of water at the protein surface.[2,5] It was shown that the formation of this network occurs via a quasi two-dimensional percolation transition of the hydration water at the protein surface.[5]

4.1.3. Regime 3 (w_1 = 0.17−0.5). Regime 3 corresponds to the appearance of the "structured" water.[12] It was concluded that the structured water consists of molecules that interact with hydrophobic surface patches on the protein while bridging between bound water molecules. Rupley and Careri[2] attributed this region to the condensation of water molecules over weakly interacting surface elements, probably nonpolar atoms not adjacent to charged and polar groups. The $\overline{H}_1^E$ values change moderately in this water content region (Figure 2). The $\overline{H}_1^E$ values vary between −46 and −43.7 kJ mol^{-1}. Probably, this region corresponds to the completion of the formation of the spanning hydrogen-bonded network of water.

Hydration-induced changes in the distribution of isotropic chemical shifts for lysozyme were obtained from the ^{13}C NMR spectra.[13,14] Solid-state ^{13}C NMR spectra of lysozyme indicate that the dried protein is characterized by a relatively broad distribution of isotropic chemical shifts. Hydration of lysozyme leads to a decrease in the distribution of conformations 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 $\overline{H}_2^E$ values are also complete within regime 3 (Figure 3A−C).

The onset of the biological activity of proteins (Figures 3 and 4) was observed in this region. Khurgin et al.[48] measured the chymotrypsin-catalyzed hydrolysis of the amide substrate *N*-succinyl-L-phenylalanine-*p*-nitroanilide at low and medium hydration levels (Figure 3). Rupley et al.[49] studied the lysozyme-catalyzed hydrolysis of the hexasaccharide of *N*-acetylglycosamine $[(GlcNAc)_6]$ (Figure 4) as a function of water content. The reactions grow sharply at w_1 > 0.15.

Quasielastic neutron and light-scattering techniques were employed to study the effect of hydration on the internal dynamics of lysozyme in the picosecond to nanosecond time range.[50] The increase of hydration level activates the fast relaxation process in regime 2 (Figure 3B). It is interesting to note that the $\overline{H}_1^E$−w_1 and $\overline{H}_2^E$−w_1 curves and the dependence of fast conformational fluctuations on the hydration level are similar to the hydration dependence of hydrogen isotope exchange (Figures 2 and 3B). The $\overline{H}_1^E$ and $\overline{H}_2^E$ values and enzymatic activity have different dependencies on hydration (Figures 3A and 4). Enzymatic activity is suppressed within regime 2 (Figure 3A and 4).

The slow relaxation process[50] was activated at w_1 > 0.15 (Figure 4). It was proposed that the slow relaxation process might be related to motions of secondary structures. The dependence of the slow process on the water content correlates with the hydration dependences of the enzymatic activity of lysozyme and the rotational relaxation time of a probe molecule close to the protein surface studied by electron paramagnetic resonance (EPR) spectroscopy[51] (Figure 4). The slow relaxation process[50] becomes active and the distribution of conformational states[13,14] is close to normal only when the hydration of the protein surface reaches a particular level. When the formation of the spanning hydrogen bond network of water is complete, the proteins can become catalytically active.

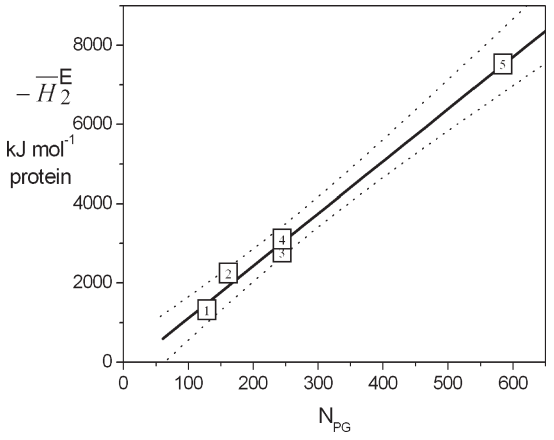

Figure 5. The $\overline{H}_2^P$ values at $w_1 = 1.0$ as a function of the number of peptide groups, N_{PG}: (1) lysozyme; (2) α-chymotrypsin (adapted data from ref 7); (3) chymotrypsinogen A; (4) β-lactoglobulin; and (5) HSA. $-\overline{H}_2^E = 210(230) + 13.2(0.7)\cdot N_{PG}$, where the number of experimental points is $N = 5$, the standard error of estimation is $S_o = 260$, and the correlation coefficient is $R = 0.996$. The dashed lines show the 95% confidence interval.

The most important observations are the correlations presented in Figures 2−4. 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 partial quantities corresponding to the protein interior are largely complete within regime 2. The changes in the partial quantities corresponding to the protein surface are complete within regime 3.

4.1.4. Regime 4. ($w_1 > 0.5$). The main features of regime 4 can be described as follows. At the highest water contents, the proteins are in a flexible (elastic) state.[1,25] Excess partial quantities reach their fully hydrated values at a water weight fraction more than 0.5 when the coverage of both polar and adjacent weakly interacting surface elements no longer changes appreciably upon further hydration.

The excess partial enthalpies, $\overline{H}_2^E$, (Figure 3A−C) reach their maximal values. The $\overline{H}_1^E$ values are close to the enthalpy of condensation of pure water at 25 °C and atmospheric pressure (−43.7 kJ mol^{-1}). Bulk water was observed in this region from proton NMR measurements.[12] Molecular motion of these water molecules is determined solely by the interaction characteristics of water molecules. This means that at $w_1 > 0.5$, water addition has no significant effect on the thermochemical excess functions. The changes of the thermochemical excess functions can mainly be attributed to changes in the state of the proteins. In other words, the protein hydration is full, and water molecules added at $w_1 > 0.5$ are not perturbed by the protein macromolecules.

The high water content region is the biologically important regime. Aggregation/association (P−P interactions) in the solutions at high water content is a subject of extensive studies.[52−57] One of the possible reasons for this process is the disruption of the crucial hydration shell in the presence of low molecular additives (for example, salts and organic solvents). One can roughly estimate 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 of H_2O/g of protein or 794 water molecules per lysozyme molecule. A monolayer water coverage was estimated from desorption calorimetry[25] (420 molecules) and proton NMR[12] (417 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,[58] the hydration shell of lysozyme may be estimated as 0.57 nm. This value is consistent with estimation presented in ref 6. It was shown that the hydration shell of proteins is 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 contents. This value is 1.14 nm.

The $\overline{H}_2^E - w_1$ curves are in agreement with the previously published results for α-chymotrypsin.[7] We compared the $\overline{H}_2^E$ values at $w_1 = 1.0$ for lysozyme, chymotrypsinogen A, β-lactoglobulin, HSA, and α-chymotrypsin with the content of various protein functional groups (charged, hydrophilic, and hydrophobic groups). The best correlation was observed with the number of peptide groups. As can be concluded from Figure 5, the intercept of this linear correlation is close to 0. This result shows that the $\overline{H}_2^E$ values are mainly determined by the state of the peptide groups, which are the most numerous in the proteins.

4.2. Enthalpy of Hydration of Proteins. Figure 6 shows how the $\Delta H_{hydr}^{H_2\,O/gas}$ values depend on the weight fraction of water. The enthalpy of hydration of proteins, $\Delta H_{hydr}^{H_2\,O/gas}$, was calculated using eqs 6 and 14. As can be concluded from Figure 6A−C, the $\Delta H_{hydr}^{H_2\,O/gas}$ functions are smooth curves. The most significant $\Delta H_{hydr}^{H_2\,O/gas}$ values were observed at the lowest water weight fractions ($\Delta H_{hydr}^{H_2\,O/gas} \approx -62.0$ (1.5) kJ mol^{-1} of water). These values are close to the $\overline{H}_1^E$ values (Figure 2) at the lowest water contents. This result is due to the fact that at the lowest water contents, the $[\overline{H}_P^h - \overline{H}_P^0]_{solid}$ component in eq 6 is close to 0 because the $\overline{H}_{Psolid}^h$ and $\overline{H}_{Psolid}^0$ values do not differ significantly. This agreement between the $\Delta H_{hydr}^{H_2\,O/gas}$ and $\overline{H}_1^E$ values supports the reliability of our calculations.

At $w_1 > 0.5$, the $\Delta H_{hydr}^{H_2\,O/gas}$ values reach saturation and are close to the enthalpy of condensation of water (−43.7 kJ mol^{-1} of water). This saturation range is consistent with that observed for the excess partial quantities (Figures 2 and 3).

To show the generality of our findings, the hydration enthalpies for lysozyme, chymotrypsinogen A, and HSA were compared with the $\Delta H_{hydr}^{H_2\,O/gas}$ values obtained from temperature dependence of water sorption isotherms[20,59] and direct calorimetric measurements.[21] As can be concluded from Figure 6A−C, a good agreement was observed between our data and the previously published results. This fact is indicative of the reliability of our experiments.

One should stress that the $\Delta H_{hydr}^{H_2\,O/gas}$−water content curves are very reproducible in different experiments and at different conditions. However, the changes on the $\Delta H_{hydr}^{H_2\,O/gas}$−water content curves describing a transition from the glass to the flexible state are not pronounced. One should explain why the thermochemical functions presented in Figures 1−3 and 6 have different profiles. The partial enthalpies, $\overline{H}_j^E$, which contain the second derivatives of G, can be defined as follows

$$\overline{H}_j^E = \left(\frac{\partial H^E}{\partial n_j}\right) = \left(\frac{\partial G}{\partial n_j}\right) - T\left(\frac{\partial^2 G^E}{\partial T\,\partial n_j}\right) \qquad (19)$$

where n_j is the molar amount of the jth component.

These second derivative quantities signify the actual thermodynamic situation of the target jth component. This contrasts with what is contained in the excess functions (for example, H^E), the first derivative quantities, which provide the respective global averages. As can be concluded from our work, the published apparent heat capacities of proteins[2,8] and the partial quantities

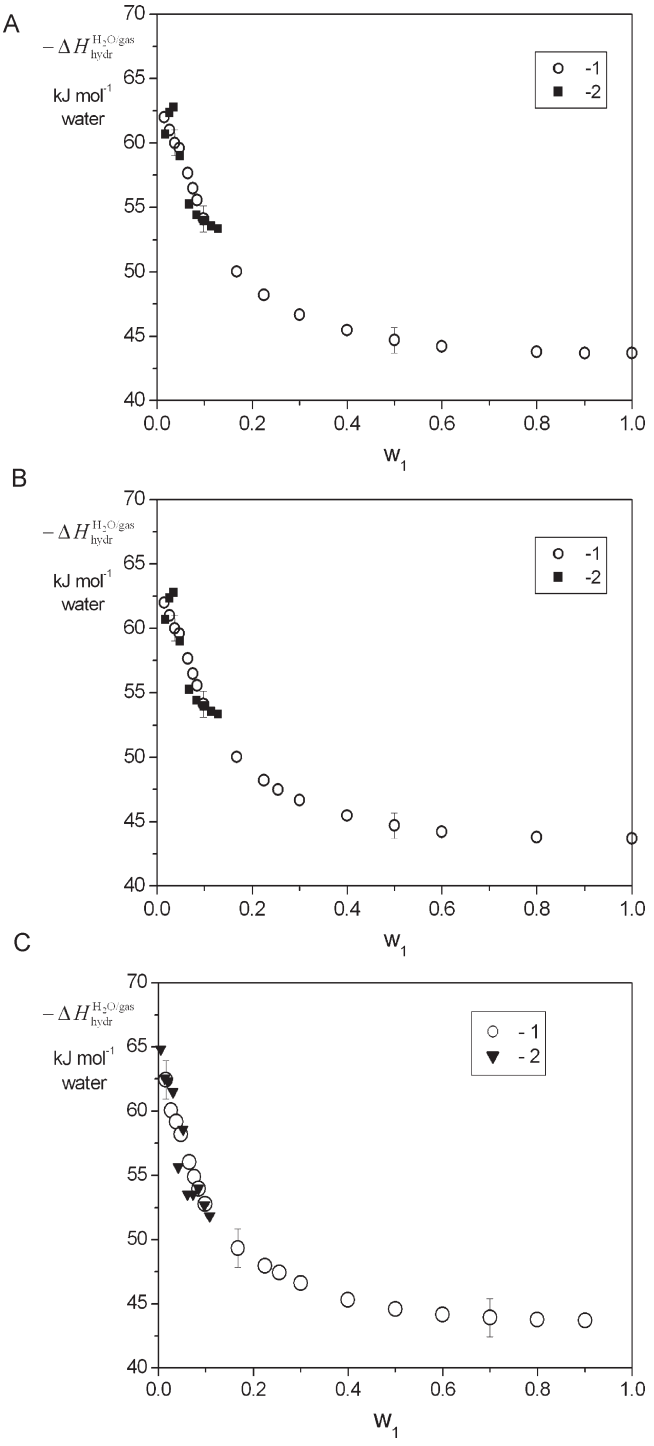

Figure 6. (A) (1) Enthalpy of hydration of lysozyme, $\Delta H_{hydr}^{H_2O/gas}$, as a function of the weight fraction of water, w_1, at 25 °C (this work). (2) Enthalpy of hydration of lysozyme, $\Delta H_{hydr}^{H_2O/gas}$, as a function of the weight fraction of water, w_1 (adapted data from ref 20 with permission of IOP science). (B) (1) Enthalpy of hydration of chymotrypsinogen A, $\Delta H_{hydr}^{H_2O/gas}$, as a function of the weight fraction of water, w_1, at 25 °C (this work). (2) Enthalpy of hydration of chymotrypsinogen A, $\Delta H_{hydr}^{H_2O/gas}$, as a function of the weight fraction of water, w_1 (adapted data from ref 59). (C) (1) Enthalpy of hydration of HSA, $\Delta H_{hydr}^{H_2O/gas}$, as a function of the weight fraction of water, w_1, at 25 °C (this work). (2) Enthalpy of hydration of bovine serum albumin, $\Delta H_{hydr}^{H_2O/gas}$, as a function of the weight fraction of water, w_1, at 20 °C (adapted data from ref 21).

obtained in this work (Figures 2 and 3) and in ref 7 show similar profiles and a glasslike transition in the w_1 range from 0.06 to 0.5. Figure 1 shows an excess function of mixing (H^E). It is the first derivative of G. Therefore, there is no transition on this curve. Hydration enthalpy presented in Figure 6 is a complicated combination of the protein and water partial quantities. Therefore, a smooth curve was observed in this case. These facts are indicative of the reliability of our calculations.

5. CONCLUSIONS

Isothermal batch calorimetry was applied to study the hydration of proteins. The hydration process was characterized by analyzing the excess partial quantities. This method allows for studying separately the protein and water excess partial quantities in the entire range of water content. The excess partial quantities are very sensitive to the changes in the state of water and the proteins. It was shown that the protein and water contributions to the excess functions depend markedly on the hydration level. At the lowest water contents, changes of the thermochemical excess functions can solely be attributed to water addition.

A transition from the glassy to the flexible state of proteins is accompanied by significant changes in the excess partial quantities of water and the proteins. This transition appears in the calculated quantities when charged groups of proteins are covered, which occurs at a water weight fraction of 0.06 and 25 °C.

Excess partial quantities reach their fully hydrated values at a water weight fraction more than 0.5 when coverage of both polar and adjacent weakly interacting surface elements no longer changes appreciably upon further hydration. At the highest water contents, water addition has no significant effect on the thermochemical excess functions. At $w_1 > 0.5$, the changes of the thermochemical excess functions can mainly be attributed to changes in the state of the proteins.

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