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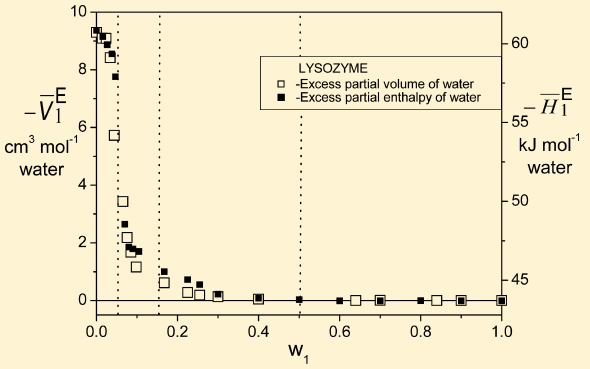
Hydration of Proteins: Excess Partial Volumes of Water and Proteins

Vladimir A. Sirotkin,* Igor A. Komissarov, and Aigul V. Khadiullina

A.M. Butlerov Institute of Chemistry, Kazan (Volga Region) Federal University, Kremlevskaya street, 18, Kazan, 420008, Russia

ABSTRACT: High precision densitometry was applied to study the hydration of proteins. The hydration process was analyzed by the simultaneous monitoring of the excess partial volumes of water and the proteins in the entire range of water content. Five unrelated proteins (lysozyme, chymotrypsinogen A, ovalbumin, human serum albumin, and β-lactoglobulin) were used as models. The obtained data were compared with the excess partial enthalpies of water and the proteins. It was shown that 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 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 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 phenomenon of considerable fundamental importance and practical interest. It is well-known that water bound to proteins (hydration or biological water) plays a crucial role in determining their stability, dynamics, and functions.[1−5] On the other hand, there are essential differences between the hydration water surrounding the protein and bulk water.[1−7] This means that a characterization of protein hydration requires elucidating the effects of both the protein on water and water on the protein.

Volumetric studies have traditionally been of great importance in ascertaining a better understanding of protein−water interactions. Below, a short review of the available studies on the hydration of proteins is given. Because our paper presents a volumetric study of the water−protein systems, a major focus of this section aims to discuss the corresponding volume changes. More comprehensive reviews have been given in refs 1−4.

Volume is an important thermodynamic quantity directly related to the compactness or globularity of the protein molecule and is generally thought to arise from a combination of factors.[8−16] The cavities and internal voids appear to represent a major positive contribution to the value of the volume change. The hydration of charged and polar groups causes a decrease in volume. On the other hand, the volume changes associated with the exposure of hydrophobic groups depend on the model compounds selected and fall into the range from small negative to positive values, and it is not clear whether the volume changes associated with the exposure of hydrophobic groups upon protein unfolding is net negative or positive and if the volume change associated with hydrophobic hydration plays an important role in the total volume change.

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.[1−4] Fullerton et al.[17] 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, (water weight fraction) ≈ 0−0.05), "polar bound" (water molecules directly hydrogen bonded to polar sites on the protein macromolecule; w_1 ≈ 0.05−0.2), "structured" (water molecules that are motionally perturbed by a protein but not bonded to it; w_1 ≈ 0.2−0.58), and bulk. Lioutas et al.[18] performed similar experiments and also found three fractions of water with motional properties different from bulk water.

This division into four steps is consistent with classifications derived from thermodynamic measurements. For example, Yang and Rupley[19] 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 = 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 of 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. Similar division

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into four regimes was observed for the excess partial enthalpies of water and proteins.[7] Since the hydration water is different from bulk water in various thermodynamic quantities like heat capacity[19] and enthalpy,[7] the volumes (densities) of the hydration and bulk water should also differ significantly.

There is much discussion in the literature concerning the evaluation/prediction of the water density (volume) in the hydration shell of the proteins. Svergun et al.[20] combined neutron and X-ray scattering to show that for lysozyme and other proteins the average density of the first hydration shell is significantly higher than that of bulk water. This finding is consistent with results from molecular simulation, crystallographic, and solution works.[8,9,14,21−23]

There is substantial literature on the partial volumes of proteins in the solutions at high water content.[3,8,9,15,16,24−26] The partial specific volumes for the majority of globular proteins fall in a narrow range between 0.70 and 0.75 $cm^3\ g^{-1}$ (Table 1). Kim and Kauzmann[27] measured the concentration

Table 1. Partial Specific Volumes of Proteins, $\bar{V}_2$,[a] at w_1 = 1.0, w_1 = 0, and 25 °C

no.	protein	$\bar{V}_2$ (w_1 = 1.0), $cm^3\ g^{-1}$	$\bar{V}_2$ (w_1 = 0), $cm^3\ g^{-1}$
1	lysozyme	0.712;[24] 0.712;[15] 0.712;[16] 0.712 (this work)	0.756 (this work)
2	chymotrypsinogen A	0.730;[8] 0.733;[16] 0.730 (this work)	0.777 (this work)
3	ovalbumin	0.746;[16] 0.746;[15] 0.7477;[25] 0.745 (this work)	0.790;[29] 0.793 (this work)
4	serum albumin	0.739;[8] 0.739;[26] 0.739 (this work)	0.800;[30] 0.796 (this work)
5	β-lactoglobulin	0.751;[15] 0.751;[16] 0.750 (this work)	0.802;[31] 0.804 (this work)

[a]The $\bar{V}_2$ values were estimated using Figure 1 and the following equation: $V^m = w_1\bar{V}_1 + w_2\bar{V}_2$.

dependences of the apparent volumes of serum albumin, ovalbumin, and oxyhemoglobin at high concentrations ($w_2 \approx$ 0.3−0.4). No effect of protein on the solvent was observed at these concentrations. This means that there is a constant partial specific volume of the solvent in the studied concentration range. Dilatometric measurements of serum albumin and oxyhemoglobin are in agreement with this conclusion.[28]

Direct measurements of the volumetric properties of binary water−protein systems at low hydration levels are relatively rare. Bull and Breese[25] (Figure 1A, curve 2), Bull and Neurath[29] (Figure 1A, curve 3), Low and Richards[30] (Figure 1B, curves 2 and 3), and McMeekin et al.[31] (Figure 1C, curve 2) measured the volumes of the protein−water systems between w_1 of 0 and 0.56. They showed that, at $w_1 > 0.2$−0.3, the volumes of the protein−water systems depend linearly on the water content. However, at the lowest w_1 values, there are deviations from the linearity. As can be concluded from these works,[25,29−31] the partial specific volumes of proteins are lower at high hydration levels than in the dried state (Figure 1A−C and Table 1). Bull and Breese[25] estimated that at $w_1 < 0.2$ the partial specific volume of water is lower than that of bulk water.

The measured volumes[25,29−31] 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 volume of the 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.[32] The partial internal energies and entropies of BPTI and water were only computed for the dry and fully hydrated protein.[32] Apparent heat capacities of lysozyme[19] and BPTI[32] 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 dry and hydrated proteins.

A novel method has recently been proposed for simultaneously studying the partial enthalpies of water and the proteins.[6,7] This method is based on the analysis of the thermodynamic functions of mixing. In this work, we applied this method for the simultaneous monitoring of the partial volumes 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 partial volumes of water and the proteins and show how these quantities correlate with coverage of the proteins by the water molecules.

Five unrelated proteins (lysozyme, β-lactoglobulin, chymotrypsinogen A, ovalbumin, and human serum albumin) were used as models. They are among the most studied and applied in protein physical chemistry.[33,34] Lysozyme is a small monomeric protein composed of 129 amino acid residues. Lysozyme is an example of an α+β protein. The physiological role of lysozyme is to hydrolyze polysaccharide chains.[33,34] 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] α-Chymotrypsinogen A is a typical globular protein. It contains 9% helix and 34% β-sheet.[37] This protein consists of 246 amino acid residues.[38] Ovalbumin is the main protein found in egg white. This protein consists of 385 amino acid residues.[39] It is presumed to be a storage protein.[40] Human serum albumin (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.[41] It consists of 585 amino acid residues. HSA is an example of a predominantly α-helix protein.

2. METHODOLOGY

The thermodynamic properties of a real binary water−protein 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):

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

The Z^m_{id} values can be calculated using eq 2:

$$Z^m_{id} = w_1 Z_1^0 + w_2 Z_2^0 \tag{2}$$

where Z_1^0 and Z_2^0 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 nonideal due to strong specific interactions between components (first of all, hydrogen bonding and charge−charge interactions).

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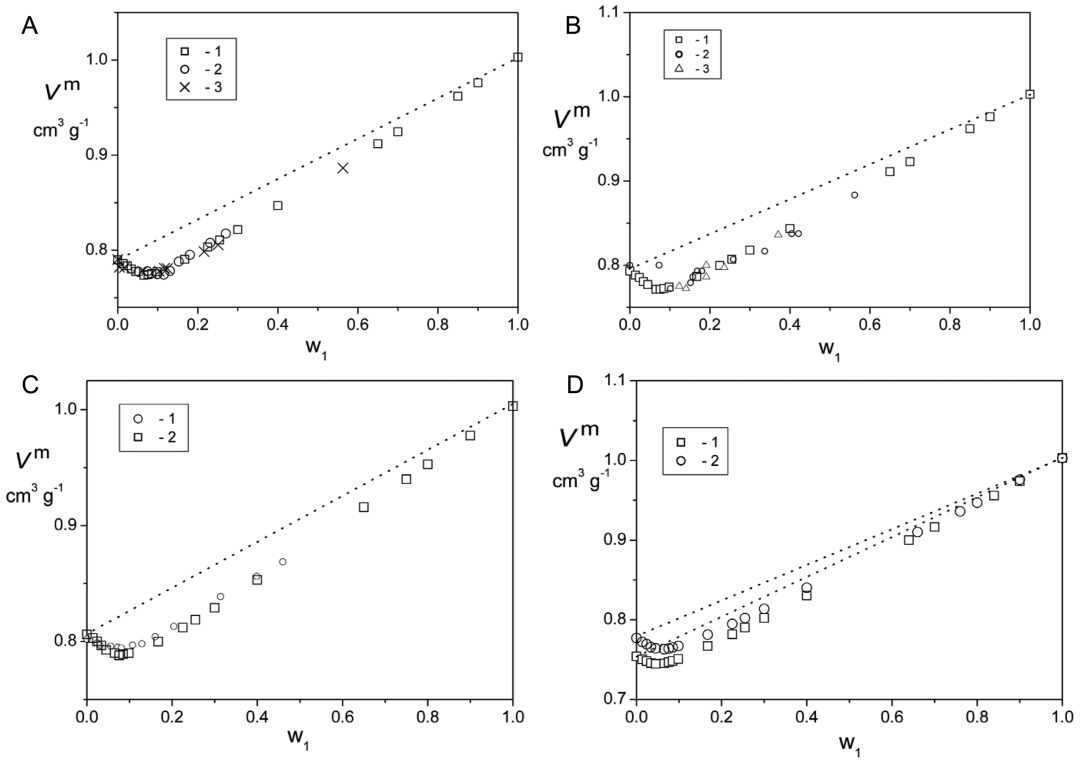

Figure 1. (A) The V^m values for the water−ovalbumin mixtures as functions of the weight fraction of water, w_1, at 25 °C: (1) This work. Standard errors of estimation of the V^m values were 0.002−0.003 cm^3 g^{-1}. (2) Adapted data from ref 25. (3) Adapted data from ref 29. The dashed line corresponds to the ideal binary mixture. (B) The V^m values for the water−human serum albumin mixtures as functions of the weight fraction of water, w_1, at 25 °C: (1) This work. (2) Adapted data from ref 30. Determined on dehydration cycle. (3) Adapted data from ref 30. Determined on hydration cycle. The dashed line corresponds to the ideal binary mixture. (C) The V^m values for the water−β-lactoglobulin mixtures as functions of the weight fraction of water, w_1, at 25 °C: (1) Adapted data from ref 31. (2) This work. The dashed line corresponds to the ideal binary mixture. (D) The V^m values as functions of the weight fraction of water, w_1, at 25 °C: (1) Lysozyme. This work. (2) Chymotrypsinogen A. This work. The dashed lines correspond to the ideal binary mixtures.

The Z^E values are composed of two components (eq 3):

$$Z^E = w_1\bar{Z}_1^E + w_2\bar{Z}_2^E \tag{3}$$

where $\bar{Z}_1^E$ is the excess partial function for component 1 (water) and $\bar{Z}_2^E$ is the excess partial function for component 2 (protein).

The excess partial volumes at 25 °C and atmospheric pressure can be calculated using eqs 4 and 5:

$$\bar{Z}_1^E = Z^E - w_2\left(\frac{\partial Z^E}{\partial w_2}\right)_{T,P} = \bar{V}_1^E \tag{4}$$

$$\bar{Z}_2^E = Z^E - w_1\left(\frac{\partial Z^E}{\partial w_1}\right)_{T,P} = \bar{V}_2^E \tag{5}$$

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

3. EXPERIMENTAL SECTION

3.1. Materials. Hen egg-white lysozyme, human serum albumin, bovine α-chymotrypsinogen A, hen egg-white ovalbumin, 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 proved by dynamic light scattering measurements (90Plus Particle Size Analyzer, Brookhaven Instruments Corporation, USA) to be more than 95%. The molecular weights of proteins were taken as 14300 Da (lysozyme), 66000 Da (HSA), 18200 Da (β-lactoglobulin), 45000 Da (ovalbumin), and 25700 Da (chymotrypsinogen A). Water used was doubly distilled.

3.2. Densitometry. Volumes of the protein−water mixtures, V^m, were calculated using eq 6:

$$V^m = \frac{1}{d_m} \tag{6}$$

where d_m is the density of the protein−water mixtures, g cm^{-3}.

At the highest water weight fractions ($w_1 \approx 0.65$−1.0), the densities of the protein−water mixtures were measured at 25 °C with a precision of 1.5 × 10^{-4} % using a vibrating tube densitometer (DMA-5000M, Anton Paar, Austria). The instrument constant was determined by calibrating the densitometer with double-distilled water of known density.

At the lowest and intermediate water weight fractions ($w_1 \approx$ 0−0.4), the volumes of the protein−water mixtures were determined using a helium pycnometer (AccuPyc 1330, Micrometrics, USA, 1.0 mL cell) at 25 °C. Helium was used as the displacement gas at an equilibration rate of 0.1 kPa min^{-1}. Ten sample runs were found sufficient for values of the volumes significant up to the third decimal place.

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 as 0.003 ± 0.002 g water g^{-1} protein by

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the Karl Fischer titration method according to the recommendations.[42]

At the lowest and intermediate water weight fractions (w_1 = 0−0.4), the protein preparations were prepared as follows. 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. A schematic representation of the experimental setup is given in ref 43. The water activity (a_w) in 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), KNO_3 (a_w = 0.94), $K_2Cr_2O_7$ (a_w = 0.98). Salts for the conditioning of the samples 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 the constant sample weight was reached.

4. RESULTS AND DISCUSSION

4.1. Partial Volumes of Water and Proteins. Figure 1 shows how the V^m values (functions of mixing per unit mass of the water−protein mixture) depend on the hydration level of the proteins. As can be concluded from Figure 1 and Table 1, the V^m and $\bar{V}_2$ values are in agreement with the previously published results for β-lactoglobulin, ovalbumin, lysozyme, chymotrypsinogen A, and serum albumin,[8,15,16,25,29−31] indicating that the apparatus and the experimental procedure are adequate to calculate the volume values.

Figure 2 shows how the V^E values (excess functions of mixing per unit mass of the water−protein mixture) depend on the hydration level of proteins. The V^E values were calculated using eqs 1 and 2. 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. Figure 2 shows that there is a contraction in the entire range of water contents. The V^E values differ essentially from zero, indicating that the water−protein mixtures are nonideal in the entire range of water contents. The most significant deviations were observed at w_1 ~0.2−0.3.

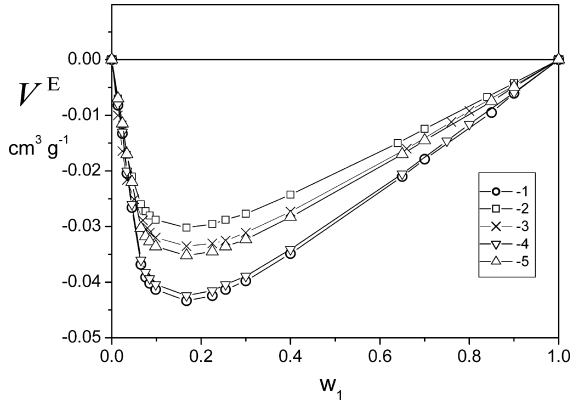

Figure 2. The V^E values as functions of the weight fraction of water, w_1, at 25 °C: (1) human serum albumin; (2) lysozyme; (3) chymotrypsinogen A; (4) β-lactoglobulin; (5) ovalbumin.

As previously discussed in refs 8−16, the volume changes of proteins accompanying different processes (including folding/unfolding or hydration/dehydration) can be described by three terms:

(i) ΔV_{int} is the intrinsic volume, which originates from the van der Waals volume of the constituent atoms plus the volume of internal cavities in a protein molecule generated by imperfect atomic packing.

(ii) ΔV_{hydr} is the hydration volume, describing the volume associated with the hydration of solvent-accessible protein atomic groups, i.e., from protein−solvent interactions around the charged (via electrostriction), polar (e.g., hydrogen-bonding), and nonpolar (hydrophobic hydration) atomic groups on the protein surface.

(iii) ΔV_{therm} is the thermal volume, which results from thermally induced mutual molecular vibrations and reorientations.

In this work, the V^E values may be explained by changes in two terms, ΔV_{int} and ΔV_{hydr}, because the ΔV_{therm} value is zero at constant temperature (25 °C).

Figures 3 and 4 present the excess partial volumes of water, $\bar{V}_1^E$, and the excess partial volumes of proteins, $\bar{V}_2^E$, as functions of the weight fraction of water. These partial quantities were calculated using eqs 4 and 5. To show the generality of our findings, the excess partial volumes of water and lysozyme were compared with the excess partial enthalpies of water and lysozyme[7] (Figures 3B and 4B). As can be concluded from Figures 3B and 4B, the excess partial enthalpies of water, $\bar{H}_1^E$, and lysozyme, $\bar{H}_2^E$, show similar behavior. The excess partial volume and enthalpy curves presented in Figures 3 and 4 can be divided into four parts.

Regime 1 (w_1 = 0−0.06). 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] 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 (Figure 5).

At the lowest water contents, the $\bar{V}_2^E$ and $\bar{H}_2^E$ values are close to zero and do not depend noticeably on the water content (Figure 4A and B). The fact that the proteins are in the glassy state explains this feature of regime 1. The $\bar{V}_2^E$ and $\bar{H}_2^E$ values are close to zero due to the fact that all the protein molecules came into contact mainly with the same protein molecules during this range of water contents.

At low water contents, the $\bar{V}_1^E$ and $\bar{H}_1^E$ values differ essentially from zero (Figure 3A and B). The $\bar{V}_1^E$ values are highly negative. The $\bar{V}_1^E$ values do not depend noticeably on the water content. The $\bar{V}_1^E$−w_1 curves do not depend noticeably on the nature of the protein (Figure 3A). These results show that, at the lowest water contents, changes of the excess functions can solely be attributed to water addition.

Hutchens et al.[46] studied the heat capacities of insulin at w_1 = 0 and 0.038 and of chymotrypsinogen A at w_1 = 0

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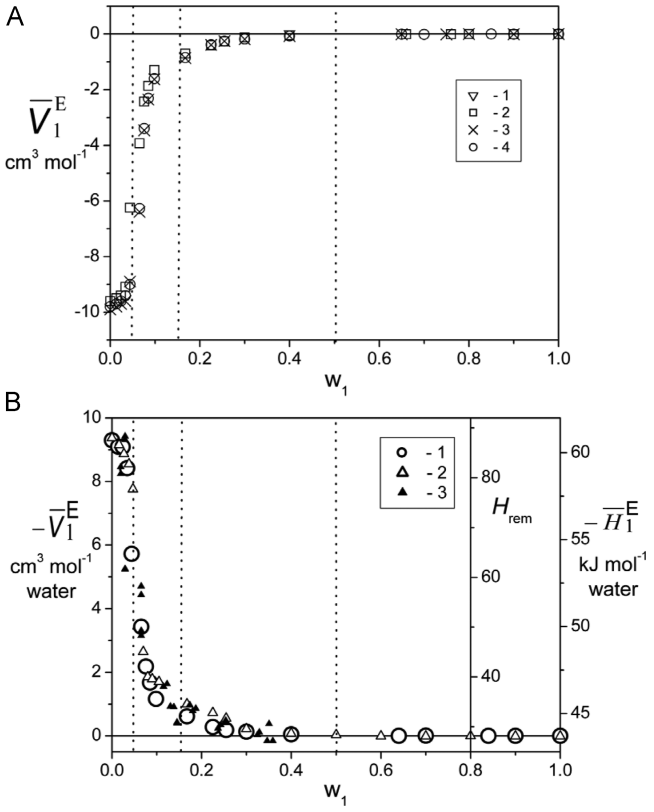

Figure 3. (A) Excess partial volumes of water, $\overline{V}_1^E$, as functions of the weight fraction of water, w_1, at 25 °C: (1) ovalbumin; (2) chymotrypsinogen A; (3) β-lactoglobulin; (4) human serum albumin. (B) Lysozyme. (1) Excess partial volume of water, $\overline{V}_1^E$, as a function of the weight fraction of water, w_1, at 25 °C: (2) Excess partial enthalpy of water, $\overline{H}_1^E$, as a function of the weight fraction of water, w_1, at 25 °C. Adapted data from ref 7. (3) 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 63.

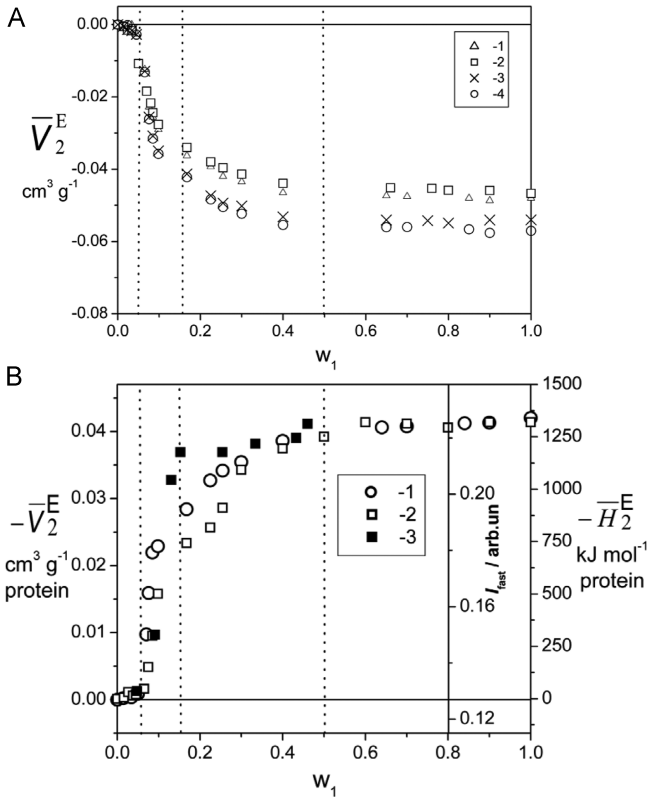

Figure 4. (A) Excess partial volumes of proteins, $\overline{V}_2^E$, as functions of the weight fraction of water, w_1, at 25 °C: (1) ovalbumin; (2) chymotrypsinogen A; (3) β-lactoglobulin; (4) human serum albumin. (B) (1) Excess partial volume of lysozyme, $\overline{V}_2^E$, as a function of the weight fraction of water, w_1, at 25 °C. (2) Excess partial enthalpy of lysozyme, $\overline{H}_2^E$, as a function of the weight fraction of water, w_1, at 25 °C. Adapted data from ref 7.

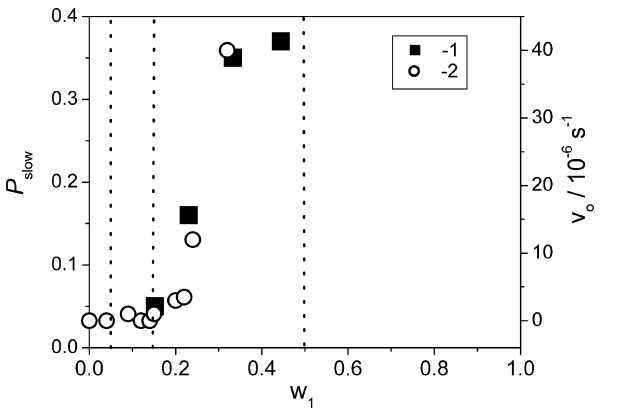

Figure 5. (1) Hydration dependence of the mobile fraction of hydrogen atoms involved in the slow relaxation process, P_{slow}. Adapted data from ref 62. (2) Enzymatic activity of lysozyme as a function of water content at pH 8 and 25 °C. Powder samples were the 1:1 $(GlcNAc)_6$–lysozyme complex. The reaction rate, v_o, was determined by product analysis. Adapted data from ref 61.

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.[1,2,17] Certain conclusions regarding the nature of the water sorption sites of the dehydrated protein can be made by analyzing the volumes of water in various organic solvents. The $\overline{V}_1^E$ values were compared with the excess partial volumes of water in organic solvents at infinite dilution in Table 2. The $\overline{V}_1^E$ values in organic solvents were estimated using eqs 1, 2, and 7:

$$V^E - x_2\left(\frac{\partial V^E}{\partial x_2}\right)_{T,P} = \overline{V}_1^E \quad (7)$$

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

Diethylamine, acetic acid, ethanol, and *N*,*N*-dimethylformamide 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. *N*,*N*-Dimethylformamide 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 2, the $\overline{V}_1^E$ $(w_1 = 0)$ values are close to that observed for diethylamine. This result suggests that, at the lowest w_1 values, the interaction with the protein elements containing the amino groups may be a dominant

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Table 2. Excess Partial Volumes of Water, $\bar{V}_1^E$, in Organic Solvents at Infinite Dilution and 25 °C

solvent	$\bar{V}_1^E$, cm^3 mol^{-1}
diethylamine	−11.0[47]
acetic acid	−4.5[48]
N,*N*-dimethylformamide	−2.77,[49] −2.7[50]
ethanol	−4.14[51]

factor controlling the state of water molecules. A similar result was obtained for the excess partial enthalpies of water.[7]

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.[19] During isothermal sorption of water, a glasslike transition results in a step on the excess partial volume and enthalpy of the proteins (Figure 4A and B). The $\bar{V}_2^E$ and $\bar{H}_2^E$ values change sharply from very low values to highly negative ones.

Regime 2 corresponds to hydration of polar groups.[1,2,17] In the water content range w_1 = 0.06−0.15, the $\bar{V}_1^E$ values change sharply from highly negative (∼ −9.8 cm^3 g^{-1}) to moderate (∼ −2.0 cm^3 g^{-1}) values (Figure 3). 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]

Regime 3 (w_1 = 0.17−0.5). Regime 3 corresponds to the appearance of the "structured" water.[17] It was concluded that the "structured" water consists of molecules which interact with hydrophobic surface patches on the protein while bridging between bound water molecules. Rupley and Careri[19] attributed this region to the condensation of water molecules over weakly interacting surface elements, probably nonpolar atoms not adjacent charged and polar groups. The $\bar{V}_1^E$ and $\bar{H}_1^E$ values change moderately in this water content region (Figure 3A and B). The $\bar{V}_1^E$ values vary between −1.0 and 0 cm^3 g^{-1}. Probably, this region corresponds to the completion of the formation of the spanning hydrogen-bonded network of water.

The partial volumes of the dried proteins are greater than the partial volumes of the proteins in the hydrated state (Table 1 and Figure 3). The partial volumes of the dried proteins are 4−5% above the values in solution. These values are consistent with the estimates presented in ref 3. Because the constitutive atomic volume of proteins does not change essentially with hydration, this result shows that the total volume of cavities in the dried proteins is greater than the total volume of cavities in the hydrated state.

It is obvious that the dehydration-induced cavities give rise to unsatisfied intermolecular forces. These forces may cause substantial rearrangements of the polypeptide chain to reduce the sizes of these cavities. In fact, the average cavity volume estimated using positron annihilation lifetime spectroscopy was essentially decreased upon transition from the flexible to the glassy-like state of lysozyme (from 0.090 nm^3 at w_1 = 0.28 (regime 3) to 0.065 nm^3 at the lowest water contents (regime 1)).[1]

Significant changes in the amide I and III regions of the dehydrated proteins were observed using Fourier transform infrared (FTIR) spectroscopy.[52−58] Klibanov and Griebenow[56] studied several proteins and showed that dehydration increases β-sheet content and lowers α-helix content. Constantino et al.[57] using FTIR spectroscopy found that the β-sheet content in lysozyme in the dehydrated state was about 44%, while in the aqueous solution it was about 18%. The dehydration-induced changes were essentially reversible.[43,56]

The dehydration-induced changes in the distribution of isotropic chemical shifts for lysozyme were obtained from the ^{13}C NMR spectra.[59,60] 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 $\bar{V}_2^E$ and $\bar{H}_2^E$ values are also complete within regime 3 (Figure 4A and B).

The onset of the biological activity was observed in this region. Rupley et al.[61] studied the lysozyme-catalyzed hydrolysis of the hexasaccharide of *N*-acetylglycosamine [$(GlcNAc)_6$] (Figure 5) as a function of water content. The reaction grows 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.[62] The increase of hydration level activates the fast relaxation process in regime 2 (Figure 4B). It is interesting to note that the $\bar{V}_1^E$−w_1 and $\bar{V}_2^E$−w_1 curves and the dependence of fast conformational fluctuations on hydration level are similar to the hydration dependence of hydrogen isotope exchange[63] (Figures 3B and 4B). The $\bar{V}_1^E$ and $\bar{V}_2^E$ values and enzymatic activity have different dependencies on hydration (Figures 3, 4, and 5). Enzymatic activity is suppressed within regimes 1 and 2 (Figure 5).

The slow relaxation process[62] was activated at w_1 > 0.15 (Figure 5). 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 (Figure 5) and the rotation relaxation time of a probe molecule close to the protein surface studied by electron paramagnetic resonance (EPR) spectroscopy.[61] The slow relaxation process[62] becomes active, and the distribution of conformational states[59,60] 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.

The most important observation is the correlations presented in Figures 2−5. 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 volumes and enthalpies corresponding to the protein interior are largely complete within regime 2. The changes in the excess partial volumes and enthalpies corresponding to the protein surface are complete within regime 3.

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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] Excess partial quantities reach their fully hydrated values at water weight fractions more than 0.5 when coverage of both polar and adjacent weakly interacting surface elements no longer changes appreciably upon further hydration.

The excess partial volumes, $\overline{V}_2^E$, and enthalpies, $\overline{H}_2^E$, of proteins (Figure 4A and B) reach their minimal values. The $\overline{V}_1^E$ and $\overline{H}_1^E$ values are close to zero (Figure 3A and B). Bulk water was observed in this region from proton NMR measurements.[17] Molecular motion of these water molecules is determined solely by the interaction characteristics of water molecules. This means, at w_1 > 0.5, water addition has no significant effect on the thermodynamic excess functions. The changes of the thermodynamic 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.

One can 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 proton NMR[17] (417 molecules) and desorption calorimetry[64] (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,[65] the hydration shell of lysozyme may be estimated as 0.57 nm. This value is consistent with the estimation presented in ref 4. It was shown that the hydration shell of proteins is 0.4−0.8 nm thick.

We compared the $\overline{V}_2^E$ values at w_1 = 1.0 for lysozyme, chymotrypsinogen A, β-lactoglobulin, HSA, and ovalbumin with the content of various protein functional groups (charged, hydrophilic, and hydrophobic groups). The best correlation was observed with the number of peptide groups (Figure 6). To show the generality of our finding, we presented a similar correlation for the $\overline{H}_2^E$ values. As can be concluded from Figure 6, the intercepts of the linear correlations are close to zero. These results show that the $\overline{V}_2^E$ and H_2^E values are mainly determined by the state of peptide groups, which are the most numerous in the proteins.

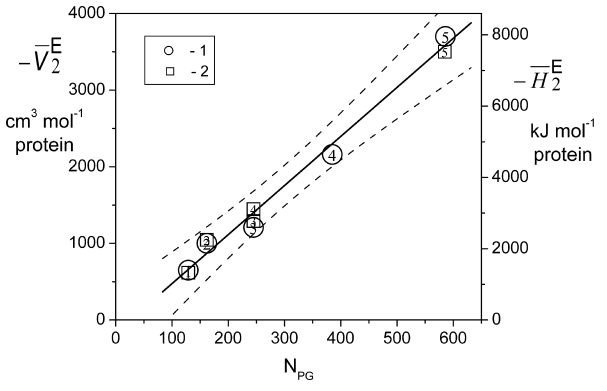

Figure 6. (1) The $\overline{V}_2^E$ values at w_1 = 1.0 as a function of the number of peptide groups, N_{PG}: (1) lysozyme; (2) β-lactoglobulin; (3) chymotrypsinogen A; (4) ovalbumin; (5) human serum albumin. $-\overline{V}_2^E$ = −160(170) + 6.4(0.5)·N_{PG}, where the number of experimental points is N = 5, the standard error of estimation is S_o = 186, and the correlation coefficient is R = 0.991. The dashed lines show the 95% confidence interval. (2) The $\overline{H}_2^E$ values at w_1 = 1.0 as a function of the number of peptide groups, N_{PG}: (1) lysozyme; (2) α-chymotrypsin; (3) chymotrypsinogen A; (4) β-lactoglobulin; (5) human serum albumin (adapted data from ref 7).

One should explain why the thermodynamic functions presented in Figures 2−4 have different profiles. The partial volumes, $\overline{V}_j^E$, which contain the second derivatives of G, can be defined as follows:

$$\overline{V}_j^E = \left(\frac{\partial V^E}{\partial n_j}\right) = \left(\frac{\partial^2 G^E}{\partial p \partial n_j}\right) \quad (8)$$

where n_j is the molar amount of 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, V^E), the first derivative quantities, which provide the respective global averages. As can be concluded from our work, the published partial enthalpies[6,7] and the partial volumes obtained in this work (Figures 3 and 4) show similar profiles and a glasslike transition in the w_1 range from 0.06 to 0.5. Figure 2 shows the excess function of mixing, V^E. It is the first derivative of G. Therefore, there is no transition on these curves. Smooth curves were observed in this case. These facts are indicative of the reliability of our calculations.

5. CONCLUSIONS

High precision densitometric measurements were applied to study the hydration of proteins. The hydration process was characterized by analyzing the excess functions of mixing. This method allows for studying separately the protein and water excess partial volumes in the entire range of water content. The excess partial volumes 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 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 volumes of water and the proteins. This transition appears in the calculated volumes when charged groups of proteins are covered, which occurs at a water weight fraction of 0.06 and 25 °C.

Excess partial volumes 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 excess functions. At w_1 > 0.5, the changes of the excess functions can mainly be attributed to changes in the state of proteins.

AUTHOR INFORMATION

Corresponding Author

*E-mail: vsir@mail.ru.

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Gregory, R. B. In *Protein-Solvent Interactions*; Gregory, R. B., Ed.; Marcel Dekker: New York, 1995; pp 191−264.

(2) Rupley, J. A.; Careri, G. *Adv. Protein Chem.* **1991**, *41*, 37−172.
(3) Kuntz, I. D.; Kauzmann, W. *Adv. Protein Chem.* **1974**, *28*, 239−345.
(4) Bagchi, B. *Chem. Rev.* **2005**, *105*, 3197−3219.
(5) Oleinikova, A.; Smolin, N.; Brovchenko, I.; Geiger, A.; Winter, R. *J. Phys. Chem. B* **2005**, *109*, 1988−1998.
(6) Sirotkin, V. A.; Khadiullina, A. V. *Thermochim. Acta* **2011**, *522*, 205−210.
(7) Sirotkin, V. A.; Khadiullina, A. V. *J. Phys. Chem. B* **2011**, *115*, 15110−15118.
(8) Chalikian, T. V.; Totrov, M.; Abagyan, R.; Breslauer, K. J. *J. Mol. Biol.* **1996**, *260*, 588−603.
(9) Chalikian, T. V.; Breslauer, K. J. *Biopolymers* **1996**, *39*, 619−626.
(10) Kharakoz, D. P. *J. Phys. Chem.* **1991**, *95*, 5634−5642.
(11) Chalikian, T. V. *Annu. Rev. Biophys. Biomol. Struct.* **2003**, *32*, 207−235.
(12) Sirotkin, V. A.; Winter, R. *J. Phys. Chem. B* **2010**, *114*, 16881−16886.
(13) Seemann, H.; Winter, R.; Royer, C. A. *J. Mol. Biol.* **2001**, *307*, 1091−1102.
(14) Voloshin, V. P.; Medvedev, N. N.; Andrews, M. N.; Berri, R. R.; Winter, R.; Geiger, A. *J. Phys. Chem.* **2011**, *115*, 14217−14228.
(15) Gekko, K.; Hasegawa, Y. *Biochemistry* **1986**, *25*, 6563−6571.
(16) Gekko, K.; Noguchi, H. *J. Phys. Chem.* **1979**, *83*, 2706−2714.
(17) Fullerton, G. D.; Ord, V. A.; Cameron, I. L. *Biochem. Biophys. Acta* **1986**, *869*, 230−246.
(18) Lioutas, T. S.; Baianu, I. C.; Sternberg, M. P. *Arch. Biochem. Biophys.* **1986**, *247*, 68−75.
(19) Yang, P.-H.; Rupley, J. A. *Biochemistry* **1979**, *18*, 2654−2661.
(20) Svergun, D.; Richard, S.; Koch, M. H.; Sayers, Z.; Kuprin, S.; Zaccai, G. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 2267−2272.
(21) Gerstein, M.; Chothia, C. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 10167−10172.
(22) Merzel, F.; Smith, J. C. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5378−5383.
(23) Merzel, F.; Smith, J. C. *J. Chem. Inf. Model.* **2005**, *45*, 1593−1599.
(24) Gekko, K.; Hasegawa, Y. *J. Phys. Chem.* **1989**, *93*, 426−429.
(25) Bull, B. H.; Breese, K. *Arch. Biochem. Biophys.* **1968**, *128*, 497−502.
(26) El Kadi, N.; Taulier, N.; Le Huerou, J. Y.; Gindre, M.; Urbach, W.; Nwigwe, I.; Kahn, P. C.; Waks, M. *Biophys. J.* **2006**, *91*, 3397−3404.
(27) Kim, K.; Kauzmann, W. *J. Phys. Chem.* **1980**, *84*, 163−165.
(28) Bernhardt, J.; Pauly, H. *J. Phys. Chem.* **1980**, *84*, 158−162.
(29) Neurath, H.; Bull, H. B. *J. Biol. Chem.* **1936**, *115*, 519−528.
(30) Low, B. W.; Richards, F. M. *J. Am. Chem. Soc.* **1954**, *76*, 2511−2518.
(31) McMeekin, T. L.; Groves, M. L.; Hipp, N. J. *J. Polym. Sci.* **1954**, *12*, 309−315.
(32) Yu, X.; Park, J.; Leitner, D. M. *J. Phys. Chem. B* **2003**, *107*, 12820−12829.
(33) Fersht, A. *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*; Freeman & Co: New York, 1999.
(34) Lehninger, A. L.; Nelson, D. L.; Cox, M. M. *Principles of Biochemistry*; Worth: New York, 1993.
(35) Eigel, W. N.; Butler, J. E.; Ernstrom, C. A.; Farrel, H. M. Jr; Harwalkar, V. R.; Jenness, R.; Whitney, R. M. *J. Dairy Sci.* **1984**, *67*, 1599−1631.
(36) Futterman, S.; Heller, J. *J. Biol. Chem.* **1972**, *247*, 5168−5172.
(37) Wang, D.; Bode, W.; Huber, R. *J. Mol. Biol.* **1985**, *185*, 595−624.
(38) Hartley, B. S. *Nature* **1964**, *201*, 1284−1287.
(39) Huntington, J. A.; Stein, P. E. *J. Chromatogr., B* **2001**, *756*, 189−198.
(40) Nisbet, A. D.; Saundry, R. H.; Moir, A. J. G.; Fothergill, L. A.; Fothergill, J. E. *Eur. J. Biochem.* **1981**, *115*, 335−345.
(41) Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153−203.
(42) Borisover, M. D.; Sirotkin, V. A.; Zakharychev, D. V.; Solomonov, B. N. In *Enzymes in Nonaqueous Solvents*; Vulfson, E. N., Halling, P. J., Eds.; Humana Press: Totowa, NJ, 2001; pp 183−202.
(43) Sirotkin, V. A. *Biochim. Biophys. Acta* **2005**, *1750*, 17−29.
(44) Nikol'skii, B. P. *Spravochnik Khimika (Chemist's Handbook)*; Goskhimizdat: Leningrad, Russia, 1963.
(45) Greenspan, L. *J. Res. Natl. Bur. Stand., Sect. A* **1977**, *81*, 89−96.
(46) Hutchens, J. O.; Cole, A. G.; Stout, J. W. *J. Biol. Chem.* **1969**, *244*, 26−32.
(47) Lampreia, I. M. S.; Dias, F. A.; Mendoca, A. F. S. S. *J. Chem. Therm.* **2004**, *36*, 993−999.
(48) Casanova, C.; Wilhelm, E.; Grolier, J.-P. E.; Kehiaian, H. V. *J. Chem. Therm.* **1981**, *13*, 241−248.
(49) Scharlin, P.; Steinby, K.; Domanska, U. *J. Chem. Therm.* **2002**, *34*, 927−957.
(50) de Visser, C.; Perron, G.; Desnoyers, E. J.; Heuvelsland, W. J. M.; Somsen, G. *J. Chem. Eng. Data* **1977**, *22*, 74−79.
(51) Ivanov, E. V.; Abrosimov, V. K.; Lebedeva, E. Yu. *J. Struct. Chem.* **2004**, *45*, 818−824.
(52) Prestrelsky, S. J.; Tedeschi, N.; Arakawa, T.; Carpenter, J. F. *Biophys. J.* **1993**, *65*, 661−671.
(53) van de Weert, M.; Harvez, P. I.; Hennink, W. E.; Crommelin, D. J. A. *Anal. Biochem.* **2001**, *297*, 160−169.
(54) Sirotkin, V. A.; Korolev, D. V. *Thermochim. Acta* **2005**, *432*, 246−253.
(55) Sirotkin, V. A.; Solomonov, B. N.; Faizullin, D. A.; Fedotov, V. D. *Russ. J. Phys. Chem. A* **2002**, *76*, 2051−2057.
(56) Griebenow, K.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10969−10976.
(57) Costantino, H. R.; Griebenow, K.; Mishra, P.; Langer, R.; Klibanov, A. *Biochim. Biophys. Acta* **1995**, *1253*, 69−74.
(58) Grdadolnik, J.; Marechal, Y. *Biopolymers* **2001**, *62*, 40−53.
(59) Kennedy, S. D.; Bryant, R. G. *Biopolymers* **1990**, *29*, 1801−1806.
(60) Gregory, R. B.; Gangola, M.; Gilpin, R. K.; Su, W. *Biopolymers* **1993**, *33*, 513−519.
(61) Rupley, J.; Yang, P. H.; Tollin, G. *ACS Symp. Ser.* **1980**, *127*, 111−132.
(62) Roh, J. H.; Curtis, J. E.; Azzam, S.; Novikov, V. N.; Peral, I.; Chowdhuri, Z.; Gregory, R. B.; Sokolov, A. P. *Biophys. J.* **2006**, *91*, 2573−2588.
(63) Schinkel, J. E.; Downer, N. W.; Rupley, J. A. *Biochemistry* **1984**, *24*, 352−366.
(64) Kocherbitov, V.; Arnebrant, T.; Söderman, O. *J. Phys. Chem. B* **2004**, *108*, 19036−19042.
(65) Chaplin, M. F. *Biophys. Chem.* **1999**, *83*, 211−223.