ISSN 0036-0244, Russian Journal of Physical Chemistry A, 2007, Vol. 81, No. 8, pp. 1341–1345. © Pleiades Publishing, Ltd., 2007.
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BIOPHYSICAL CHEMISTRY

Hydration–Dehydration of Human Serum Albumin Studied by Isothermal Calorimetry and IR Spectroscopy

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Received June 5, 2006

Abstract—Based on a comparison of the data on the isothermal calorimetry of the interaction of human serum albumin with water and the adsorption isotherms of water vapor on the protein obtained by IR spectroscopy, an experimental method was used for the first time to study the thermochemical and sorption characteristics of protein hydration–dehydration over the entire range of the thermodynamic activities of water. A mechanism was proposed to explain the relationships between the thermochemical properties, protein water content, and the moistening method.

DOI: 10.1134/S0036024407080298

Protein hydration is a phenomenon of fundamental importance and practical interest. It is well known that water plays a key role in the biological functions of proteins [1, 2]. The interaction of proteins with water is also of importance for the formation of their native spatial structure.

Many properties of proteins depend on the moistening method. In particular, a hysteresis phenomenon is characteristic of the adsorption of water vapor on proteins. In this case, a desorption branch lies above a sorption branch [1, 3, 4]. On the other hand, a new area of science, enzymatic catalysis in organic media, has been intensely developed [5–7]. Studies in this area strongly suggest that protein catalysts in organic media can catalyze industrially important synthetic reactions (such as peptide synthesis and esterification), and protein adsorbents can selectively bind low-molecular-weight compounds. However, the efficiency of these catalysts and adsorbents essentially depends on the moistening method. In particular, Ke and Klibanov [8] found that the catalytic activity of enzymes in organic media was much higher when the biocatalyst preparation procedure involved a dehydration stage. In general, this means that a study of the physicochemical characteristics of protein hydration–dehydration is of considerable current interest and practical importance.

Efficient analytical techniques capable of providing thermodynamic information on intermolecular protein–water interactions should be used in order to understand the character of protein hydration. IR spectroscopy is a technique of this kind. This technique has been successfully used to study the hydration of solid protein sorbents [1, 2, 9]. Thus, in particular, Careri et al. [10] measured the isotherms of D_2O adsorption on lysozyme at 300 and 311 K. Our research group developed an experimental method for measuring the sorption–desorption isotherms of H_2O vapor on protein films. The efficiency of this method was demonstrated by Sirotkin [11, 12] using the bovine pancreatic α-chymotrypsin enzyme as an example.

Isothermal calorimetry was found to be an effective technique that provided direct thermodynamic information on the interaction of proteins with water in various environments. Thus, in particular, Amberg [13] performed the direct calorimetric determination of the heat effects of adsorption of water vapor on bovine serum albumin over the range of water activities from 0 to 0.6. Smith et al. [14] calorimetrically measured the heats of water adsorption on lysozyme over the range of relative water-vapor pressures from 0 to 0.895. Kocherbitov et al. [15] used sorption calorimetry to measure the adsorption isotherm of water on lysozyme and the corresponding heat effects over the entire range of water activities. However, the desorption branch was characterized only over the range of water activities from 0.53 to 1.0.

In this work, we were the first to study the hydration–dehydration of proteins over the entire range of the thermodynamic activities of water based on a comparison between the data obtained using isothermal calorimetry and IR spectroscopy. The aim of this work was to develop an experimental method for studying the thermochemical and sorption characteristics of the hydration–dehydration of proteins over the entire range of the thermodynamic activities of water.

A transport protein, human serum albumin, was chosen as a test material. This is a much studied protein, whose amino acid composition, spatial structure, and function mechanism are well understood [16]. It is widely used in the physicochemical studies of the behavior of proteins in both aqueous solutions and organic environments.

EXPERIMENTAL

Materials. Commercial human serum albumin (Sigma, A 1887; protein content, no less than 96–99%; fatty acid content, no higher than 0.005%) was used without further purification. Twice-distilled water was used.

Isothermal calorimetry. Heat effects were measured at 298 K on a BT-2.15 Setaram calorimeter. The instrument was calibrated against electric current using the Joule effect. To measure the heat effects of the protein solution in water, a weighed portion of the protein (5–10 mg) was initially placed in a titanium container with Teflon gaskets. The hermetically sealed container was arranged in a calorimetric cell of the instrument; the cell was preliminarily filled with a solvent (4.0 ml). After thermal equilibration, the Teflon gaskets were pierced and the solvent interacted with the protein. Procedures used for the measurement of heat effects were described in detail elsewhere [17, 18].

The dried protein preparation was kept at 298 K and 0.1 Pa in an MGDTD-17S Setaram microthermoanalyzer until a constant sample weight was attained. The humidity of the dried protein was $0.2 \pm 0.1\%$ (g water/g protein). To obtain a thermochemical sorption isotherm, protein preparations with varied humidity were placed in a desiccator over saturated aqueous solutions of salts (LiCl, CH_3COOK, $CaCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaCl, $BaCl_2$, and $K_2Cr_2O_7$) at 298 K for a week.

The construction of thermochemical desorption isotherms of protein preparations includes the following two stages: the exposure of the protein in a desiccator over a saturated aqueous solution of $K_2Cr_2O_7$ (water activity of 0.97) and the exposure of the protein over saturated aqueous solutions of salts (LiCl, CH_3COOK, $CaCl_2$, K_2CO_3, $Mg(NO_3)_2$, NaCl, and $BaCl_2$), which gave lower vapor pressures. The activities of water over saturated salt solutions were taken from handbook [19].

IR spectroscopy. The IR spectra were measured at 298 K on a Vector 22 Fourier transform IR spectrometer (Bruker) over the range 4000–1000 cm^{-1} with a resolution of 4 cm^{-1}; the number of scans was 256. The experimental procedure was considered in detail elsewhere [11, 12, 18]. The samples were optically transparent protein films prepared by the evaporation of 25 μl of a 2% aqueous protein solution on a CaF_2 cell window in air at room temperature and humidity. The resulting film was placed in a hermetically sealed chamber with fluorite windows. The sample was dehydrated immediately in the spectrometer with the use of a flow of air dried over phosphorus pentoxide to constant absorbance in the region of the absorption band of sorbed water at 3500 cm^{-1}. The relative pressure of water vapor over phosphorus pentoxide at 298 K was <0.01 [20]. The spectrum of this sample was used as a reference in the construction of difference spectra. Next, the sample was exposed to pure water vapor. A flow of air was passed through a saturator with water and the cell with the protein. The activity of water in the vapor phase was regulated by changing the temperature difference between the measuring cell and the saturator. Data on water vapor pressures at various temperatures were taken from handbook [19].

The adsorption of water was determined from the absorption spectrum at 3500 cm^{-1} using the equation

$$h = 2.3 S_w \varepsilon_P / B_w D_P, \quad (1)$$

where h is the protein humidity (mol water/mol protein), S_w is the area of the absorption band of water (cm^{-1}), ε_P is the molar absorption coefficient at a maximum of the protein amide I band (l mol^{-1} cm^{-1}), B_w is the integrated molar absorption coefficient of water (l mol^{-1} cm^{-2}), and D_P is the absorbance at a maximum of the protein amide I band. For water, the integrated molar absorption coefficient $B_w = 96000 \pm 1000$ l mol^{-1} cm^{-2} [21] was used. The molar absorption coefficient of human serum albumin was determined based on the measured spectrum of the protein with a known concentration in heavy water, $\varepsilon_P = 237600 \pm 300$ l mol^{-1} cm^{-1}. The molecular weight of human serum albumin was taken equal to 66000.

Equation (1) was used in this work based on the following facts: Although the absorption of water by the protein occurs at various hydrophilic groups, we found that the contour shape of sorbed water was independent of the degree of humidity of the protein and similar to the contour shape of liquid water at 25°C. The IR spectra of liquid water at 25°C were reported previously [21]. This means that the molecules of sorbed water in the test protein films exhibit a broad but continuous and smooth energy distribution similar to that in liquid water. Taking into account this fact, we believe that protein films can be considered as a solid solution in which both hydrophilic and hydrophobic regions occur. Water is primarily accumulated in hydrophilic regions. According to our hypothesis, the area of the absorption band due to sorbed water S_w is directly proportional to the thickness of this solution and the concentration of water in it.

The reliability of Eq. (1) for the determination of the hydration of serum albumin was supported by a comparison with published data obtained by Bull [22] using an independent gravimetric method:

$$h(\text{IR spectroscopy}) = 0.99(0.02)h(\text{gravimetry}) - 0.01(0.02), \quad (2)$$

where the coefficient of correlation is $R = 0.99$, the standard deviation is $s_0 = 0.005$, the number of experimental points is $N = 11$, and h is the protein hydration (g/g protein).

The slope of Eq. (2) is close to unity, and the free term is close to zero. This result suggests that the quantitative determination of protein hydration can be reliably performed using Eq. (1) over the entire range of water activities.

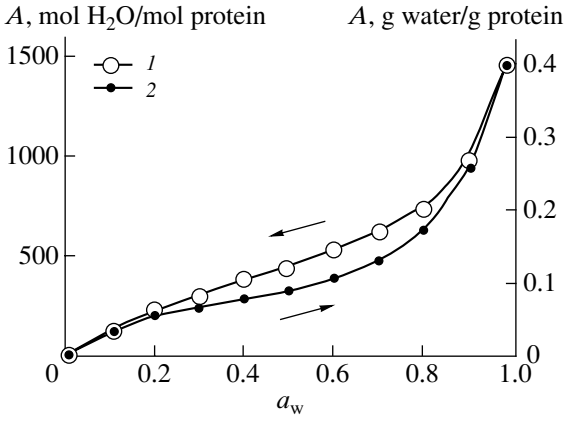

Fig. 1. Adsorption isotherms of water vapor on serum albumin: (*1*) desorption branch and (*2*) sorption branch. Solid lines were drawn by approximating adsorption isotherms with a set of polynomial expressions. Experimental errors in the determination of the moisture content of protein were 0.003–0.005 g water/g protein; *A* is the adsorption of water; a_w is the activity of water.

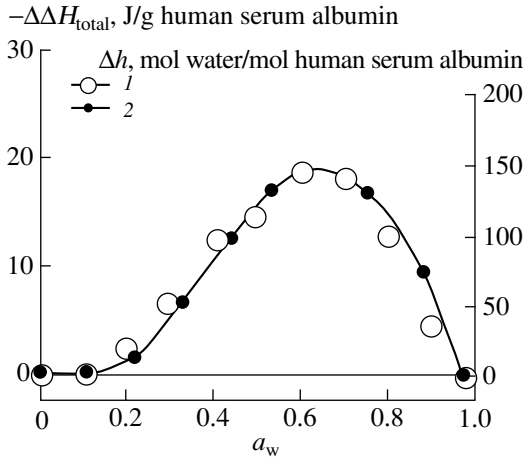

Fig. 2. (*1*) Difference ($\Delta\Delta H_{total}$) between the desorption and sorption branches of the thermochemical adsorption isotherm of water on the protein. (*2*) Difference (Δh) between the desorption and sorption branches of the adsorption isotherm of water on serum albumin.

Analogous results were obtained previously [11, 12] for bovine pancreatic chymotrypsin.

RESULTS AND DISCUSSION

Water Adsorption Isotherms

Figure 1 shows the adsorption isotherms of water vapor on serum albumin. In the study of water sorption, a protein preparation dried in a flow of air at a water activity of <0.01 served as a starting state. A protein preparation humidified at a water activity of 0.98 served as a starting state for studying the desorption of water. All of the adsorption isotherms of water were characteristically S-shaped; this is consistent with the published data [1–4].

The hysteresis phenomenon is typical of the adsorption isotherms of water vapor on proteins. In this case, the desorption branch lies above the sorption one [1, 3, 4]. As can be seen in Fig. 2, a detectable sorption hysteresis also occurs in this case. In the region of low water activities ($a_w < 0.2$), the sorption and desorption isotherms are close to each other. The greatest differences are observed in the region of medium water activities. At the highest activities of water, the difference sorption values did not significantly differ from zero.

Heat Effects of Protein Hydration–Dehydration

The heat effects (ΔH_{total}) of serum albumin hydration–dehydration were determined in the following manner: The initial state was the protein dried in a flow of air at a water activity of 0.01 and 298 K. The final state was the protein equilibrated with water vapor at varied activities, which was transferred from pure liquid water at 298 K and 1.01×10^5 Pa.

The value of ΔH_{total} (kJ/mol protein) is the difference of the partial molar enthalpies of the protein in the system with a varied activity of water ($\overline{H_p}$) and the protein in the dried state ($\overline{H_p^\circ}$)

$$\Delta H_{total} = \overline{H_p} - \overline{H_p^\circ}. \quad (3)$$

In this work, the values of ΔH_{total} were determined from the data obtained by isothermal calorimetry using the equation

$$\Delta H_{total} = \Delta H_s^\circ - \Delta H_s, \quad (4)$$

where ΔH_s° is the enthalpy of solution of the dried protein in water at 298 K (kJ/mol protein). This value was determined previously [23]: –6059 ± 185 J/mol or –91.8 ± 2.8 J/g human serum albumin; ΔH_s is the enthalpy of solution in water of the protein humidified by keeping over saturated aqueous salt solutions (kJ/mol protein).

Figure 3 shows the values of ΔH_{total}. The values of ΔH_{total} are exothermic over the entire range of water activities. The shape of the thermochemical isotherms of serum albumin hydration–dehydration is consistent with sorption relationships (Fig. 1). As in the case of water adsorption isotherms, three segments can be recognized in the thermochemical curves. Along the first segment in the region of the lowest water activities from 0 to 0.2, a dramatic decrease in ΔH_{total} is observed. Next, the heat effects exhibit a plateau over the activity range from 0.2 to 0.7. Finally, a noticeable decrease in ΔH_{total} takes place at the highest water activities.

The effect of the protein moistening method on ΔH_{total} was characterized by difference thermochemical values ($\Delta\Delta H_{total}$). As can be seen in Fig. 2, the shape of a difference thermochemical curve is consistent with

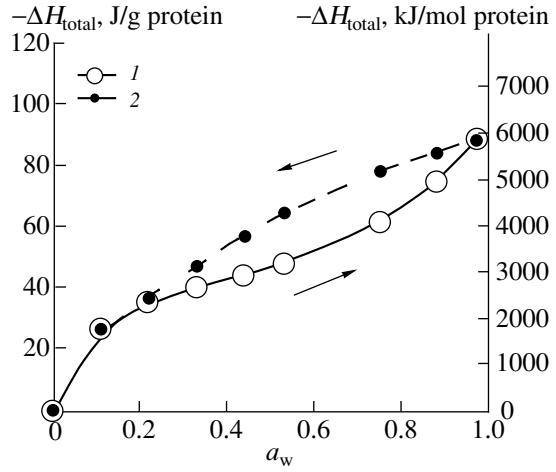

Fig. 3. Heat effects (ΔH_{total}) of the interaction of serum albumin with water: (*1*) sorption branch and (*2*) desorption branch. Experimental errors in the determination of ΔH_{total} were 3–4 J/g protein. Each measurement was performed three or more times.

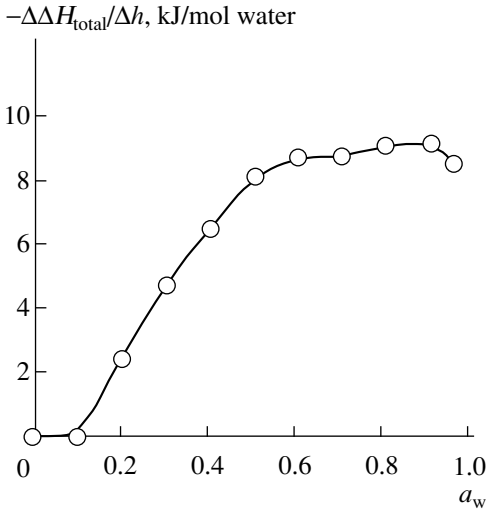

Fig. 4. Excess enthalpy of water adsorption ($\Delta\Delta H_{total}/\Delta h$) as a function of water activity.

the shape of a difference sorption isotherm. Below a water activity of 0.2, the heat effects of protein hydration and dehydration were similar. The most significant difference between the heat effects of protein hydration and dehydration were observed in the region of medium water activities. At the highest water activities, the difference thermochemical values did not significantly differ from zero.

Effect of Hydration–Dehydration on the Thermochemical and Sorption Properties of Serum Albumin

The effect of hydration–dehydration on the thermochemical and sorption properties of serum albumin can be explained based on the hypothesis that strong intermolecular contacts occur in the dried protein. This hypothesis was proposed previously [11, 12, 24]. According to this hypothesis, intermolecular contacts are formed in the dehydration of proteins mainly through hydrogen bonds and/or ionic bridges between the polar groups of the protein. These processes result in the formation of a more rigid protein structure; however, it is noticeably distorted compared with the native structure.

According to the model proposed, the first step of the adsorption process (a_w = 0–0.2) is the insertion of water molecules into the structure of a dried sorbent and the hydration of accessible sorption sites. In this region, the most significant changes in ΔH_{total} were observed (Fig. 3), which reflect the interaction of water with strongly binding sorption sites in the protein. At this step, the sorption mechanism implies the sorbate–sorption site interaction without changing the number of sorption sites. Correspondingly, in the region of low water activities, sorption and desorption curves are close to each other (Figs. 1, 3).

Considerable differences between sorption and desorption relationships were observed in the water activity range 0.2–0.9. It is believed that the second step of the adsorption process involves the rupture of interprotein contacts and the generation of new sorption sites. As a result, the number of accessible sorption sites is much greater in the course of serum albumin dehydration. Thus, in the course of protein dehydration at a water activity of 0.6, the number of sorbed water molecules was greater by 145 than that in hydration.

At the highest activities of water ($a_w > 0.9$), the process of hydration and the rupture of interprotein contacts was complete. Correspondingly, the sorption and desorption relationships were close to each other (Figs. 1, 3).

We characterized the energetics of the process that accompanied the interaction of an excess amount of water with the protein in the course of dehydration by the ratio between the values of $\Delta\Delta H_{total}$ and Δh. Figure 4 shows the dependence of the excess enthalpy of water adsorption $\Delta\Delta H_{total}/\Delta h$ on the activity of water. As can be seen in Fig. 4, the excess enthalpy approached zero at the lowest activities of water. Above a water activity of 0.2, a dramatic increase with saturation was observed above a water activity of 0.6. The maximum value of the excess enthalpy was –9.4 kJ/mol water. This result suggests that the state of an excess amount of water sorbed on the protein in the course of dehydration differs from pure water in properties. In this case, the heat effect would be close to zero. The excess enthalpy is consistent with the enthalpy of a hydrogen bond between water and the carbonyl group of acetone (–10.25 kJ/mol) [25]. Note that the number of peptide carbonyl groups in a serum albumin molecule

(585 [16]) is the highest among all of the functional groups. On the other hand, a considerable deficiency of proton-donor groups occurs in the dried protein. With consideration for these facts, the found similarity between the excess enthalpy and the enthalpy of formation of a hydrogen bond between water and acetone supports the hypothesis that an excess amount of water is primarily sorbed at the peptide carbonyl groups of the protein, which were inaccessible in the course of the sorption process.

Thus, in this work, based on a shape analysis of difference thermochemical and sorption isotherms, we found that the sorption hysteresis in the hydration–dehydration of human serum albumin primarily depends on the following processes:

(1) The formation of new interprotein contacts in the course of protein drying. This process results in a decrease in the number of accessible sorption sites in the course of protein hydration at the lowest activities of water.

(2) The activating effect of water. In the course of the sorption process, water molecules hydrate and rupture interprotein contacts to result in the appearance of new sorption sites. A maximum activating effect was reached at $a_w = 0.6$.

ACKNOWLEDGMENTS

We are grateful to B.N. Solomonov for his technical assistance.

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