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Finding of an unexpected thermal anomaly at very low temperatures due to water confined within a globular protein, bovine serum albumin

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Abstract

Heat capacities and enthalpy relaxation rates of completely anhydrous bovine serum albumin (BSA) and hydrous BSA with 1.85% (w/w) water were measured by using an adiabatic calorimeter to examine the thermal behavior at low temperatures of a small amount of water left within the globular protein molecule. The heat capacities of the hydrous BSA were larger a little on account of the presence of water molecules. In addition, two new phenomena appeared for the hydrous BSA while not for the anhydrous one: spontaneous exothermic and endothermic effects depending on the pre-cooling rates were observed and interpreted as due to a glass transition. Anomalous behavior of heat capacities was found in 60–140 K and recognized as potentially originating from a kind of phase transition. It is suggested that the water left a little within the BSA is responsible for both the phenomena occurring at low temperatures.
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Keywords: BSA; Water; Glass transition; Phase transition; Heat capacity

1. Introduction

Protein without water is in the solid state and its motion is frozen at room temperature. It functions only in the presence of water. Water here provides the protein molecule with a field for its motion and reaction. The structure and properties of protein–water systems are, in this sense, one of important subjects to be studied. Water itself is, however, one of substances showing peculiar properties: the density, for example, exhibits its maximum at 3.98 °C and decreases both with increasing and decreasing temperature. The peculiarity is attributed to the behavior on the low temperature side, namely, the property of water to form hydrogen-bond network extensively and cooperatively with decreasing temperature [1]. Bulk water ordinarily freezes into ice according to the development of the network at low temperatures, and therefore its behavior is not well known below about 240 K [1]. Water molecules in ice are hydrogen-bonded tetrahedrally with others, forming a neat three-dimensional network of the bonds. The hydrogen-atom positions within the network are frozen in the disordered arrangement as a glass transition at 100 K in bulk ice [2]. Only in the ice doped with alkali hydroxide of a certain amount, the hydrogen atoms are arranged in the ordered way below a phase transition at ca. 72 K [3,4].

On the other hand, thermal properties of globular proteins as focused on presently have been investigated concerning various phenomena in a wide temperature range by calorimetric approaches. Denaturation is the most popular subject of the investigation [5–9]. In the systems with a large amount of water, water not only surrounds but also goes into pro-

Abbreviations: BSA, bovine serum albumin; T_g, glass transition temperature; T_d, denaturation temperature; C_p, heat capacity; dH/dt, enthalpy relaxation rate; dT/dt, temperature drift rate; C_{total}, gross heat capacity of the cell; m_{BSA}, mass of the BSA; τ, relaxation time

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tein molecules, enabling the protein molecules to move and function. The denaturation temperature (T_d) is located above room temperature even in such systems, and rises remarkably as the moisture content is reduced. Therefore the measurement on T_d of the dry globular protein should be done at high temperatures reaching even 500 K close to its decomposition temperature [9]. The crystallization of water at sub-zero temperatures in protein–water systems is another subject of the investigation concerning the motion of proteins and the interaction between water and protein molecules. When cooled below room temperature, some of the water molecules form bulk ice and the others remain unfrozen with making hydrogen bonds with protein molecules on their surface. The quantity of the unfrozen water is related to the hydration structure of globular protein, and has been investigated from the relation between the water content and the amount of crystallized water [10–15]. The dynamic properties of protein–water systems have been also studied: two kinds of glass transitions have been observed. One is known to occur at low temperatures around 160–200 K regardless of kinds of globular proteins [12–15]. The transition is ascribed essentially to freezing of the motion of water molecules, and it has been often discussed that the motion of protein is associated with the dynamics [12–15]. Another glass transition has been observed at higher temperatures [8,9]. It is recognized as the freezing of the rearrangement motion corresponding to the structural change of protein molecules. Since the motion of the protein proceeds more easily in the presence of water, the glass transition temperature rises with decrease in the moisture content with the same tendency as T_d [9].

The thermal properties have barely been studied in protein–water systems with a small amount of water. The reason is that the motion of protein molecules is frozen even at room temperature and no glass transition phenomenon has been found at sub-zero temperatures in such systems so far. Such situations, however, do not mean that any rearrangement motion is suppressed even at room temperature. There is a possibility that the small amount of water left within protein molecules can move at and below room temperature, and it should be noted whether the water shows any freezing/ordering phenomenon or not. The knowledge is completely lacking but helps understand the role of water included within protein molecules and the static and dynamic properties of an aggregation of water molecules of a small size. In the present study, heat capacities and enthalpy relaxation rates of completely anhydrous bovine serum albumin (hereafter abbreviated as BSA) and the BSA with 1.85% (w/w) water were measured by using an adiabatic calorimeter. The water in the latter material is expected to remain inside of the protein molecule with forming rather strong hydrogen bonds with parts of the BSA molecule. Two novel phenomena were found as attributed to the water left a little within protein molecules; a glass transition at 155 K and an unexpected thermal anomaly at around 120 K.

2. Experiment

BSA (fraction V) was purchased from SIGMA Co. Ltd., and analyzed by a Karl-Fisher titration method to contain 1.85% (w/w) water. The BSA was prepared by freeze-drying the aqueous solution, and thus the water remaining is expected to be included within the globular protein BSA. The hydrous BSA was used as a sample as it was purchased. The BSA sample without water was prepared by drying it under vacuum for 7 days and analyzed to contain no water. An amount of 66267 g mol^{-1} was taken as the average molecular weight of BSA in the present [16].

The BSA sample was loaded into a calorimeter cell under an atmosphere of helium gas. The mass of the sample used was weighed to be 3.33 and 5.92 g for the BSA with and without water, respectively. Heat capacities were measured by an intermittent heating method using an adiabatic calorimeter [17]. The processes of energy input and thermometry were repeated under essentially adiabatic conditions. The inaccuracy and imprecision of the heat capacities obtained were estimated previously to be less than ±0.3 and ±0.06%, respectively [17].

In general, glass transition or first-order phase transition processes proceed with spontaneous heat evolution or absorption phenomena. Such phenomena are observed, by the adiabatic calorimetry, as the corresponding spontaneous temperature rise or fall, respectively, of the calorimeter cell in the thermometry period. The temperature drift rate (dT/dt) was transformed to the rate of spontaneous enthalpy relaxation (dH/dt) per gram of BSA through a following expression

$$-\frac{dH}{dt} = C_{\text{total}}\frac{dT/dt}{m_{\text{BSA}}}, \quad (1)$$

where C_{total} is the evaluated gross heat capacity of the cell and m_{BSA} is the mass of the BSA used. A minus sign on the left-hand side indicates that the spontaneous temperature rise/fall correspond to the enthalpy decrease/increase, respectively, of the sample.

The chronological order of the series of measurements and the annealing treatments is given for the anhydrous and hydrous BSA in Fig. 1(a) and (b), respectively. The anhydrous sample was, at first, cooled from room temperature down to 80 K within the calorimeter cryostat, and the first series of measurements was started thereat and ended at 300 K. The second and third series of measurements were carried out in 15–200 and 60–300 K, respectively. The sample was then annealed at 400 K for 20 h. The measurements of the fourth series were done in 60–300 K. The hydrous sample as purchased was also cooled from room temperature down to 80 K within the calorimeter cryostat, and the first series of measurements was started thereat and ended at 300 K. The second series of measurements was started after cooling from the 300 K down to 15 K and ended at 200 K. The third series was started at 60 K and ended at 300 K. The sample was then annealed at 400 K for 20 h. The measurements of the fourth, fifth, and sixth series were carried out in

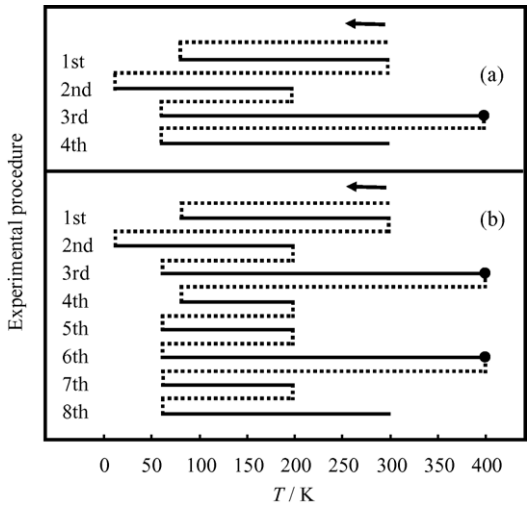

Fig. 1. The chronological order of series of heat capacity measurements and 400 K annealing treatment for anhydrous (a) and 1.85% (w/w) hydrous BSA (b). Solid and dotted lines represent each series of measurements carried out in the heating direction and the cooling treatment, respectively. Large dots at 400 K indicate that the sample was annealed for 20 h there.

Table 1
Rates of the cooling in 170–110 K immediately before each series of heat capacity measurements

Anhydrous BSA								
Series no.	1	2	3	4				
dT/dt (K min^{-1})	2	0.02	10	10				
Hydrous BSA								
Series no.	1	2	3	4	5	6	7	8
dT/dt (K min^{-1})	2	0.02	10	2	10	0.02	1	0.02

80–200, 60–200, and 60–300 K, respectively. The sample was again subjected to 20 h annealing at 400 K, and then cooled down to 60 K. The seventh and eighth series of measurements were done in the ranges 60–200 and 60–300 K, respectively. Average rates of the cooling in the range 170–110 K immediately before each series of measurements are given in Table 1. Below 110 K, the rough rates were 5 K min^{-1} in 110–90 K, 1 K min^{-1} in 90–80 K, 0.5 K min^{-1} in 80–70 K, and 0.1 K min^{-1} in 70–60 K in all the cases.

3. Results and discussion

3.1. Heat capacities before and after annealing at 400 K

Fig. 2 shows the heat capacities per mole of BSA for the anhydrous and 1.85% (w/w) hydrous BSA as prepared. The heat capacities of the hydrous BSA are larger a little on account of the presence of water molecules. The respective temperature dependences are apparently smooth. The samples were then annealed at 400 K. The 400 K was confirmed, based on the preliminary experiment of differential scanning calorimetry, to be below T_d of the present samples from the relation between moisture content and T_d of BSA. It is expected that the water left within protein molecules disperse during the

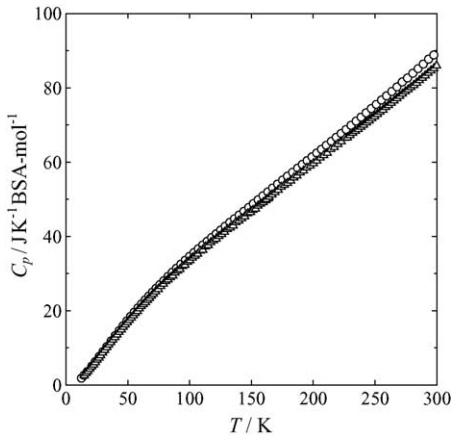

Fig. 2. Heat capacities of anhydrous (△, the second and third series of measurements) and 1.85% (w/w) hydrous BSA (◯, first and second).

annealing. The effect of the annealing on the heat capacities is shown in Fig. 3, as a typical result, in 270–300 K on an enlarged scale for the anhydrous (a) and 1.85% (w/w) hydrous BSA (b). The annealing brings definitely the values to increase for both the anhydrous and hydrous BSA. The magnitude of the increase is essentially the same between the two samples and becomes large as the annealing time is elongated. Although not shown here, it was confirmed that the heat capacities increased essentially in the whole temperature range. Since the increase occurred for the anhydrous BSA as well with the same magnitude, it is indicated firstly that the increase does not originate only from the change in the state of water. Considering that the T_d is higher than 400 K for the present samples as mentioned above, secondly, the increase would be ascribed to partial change of the BSA structure such as from helix to coil ones in parts of the molecule. The vibrational degrees of freedom of the molecule are expected to be more liberated in somehow more disordered structures to increase their contributions to heat capacity.

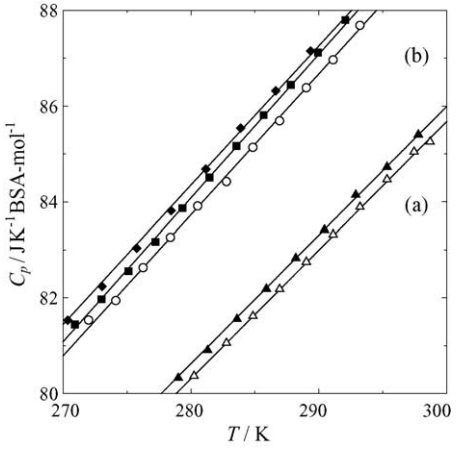

Fig. 3. Heat capacities of anhydrous (a) and 1.85% (w/w) hydrous BSA (b) on an enlarged scale in 270–300 K: △ (the first series of measurements), ◯ (first), before the 400 K annealing; ▲ (fourth), ■ (sixth), after the first annealing; ♦ (eighth), after the second annealing.

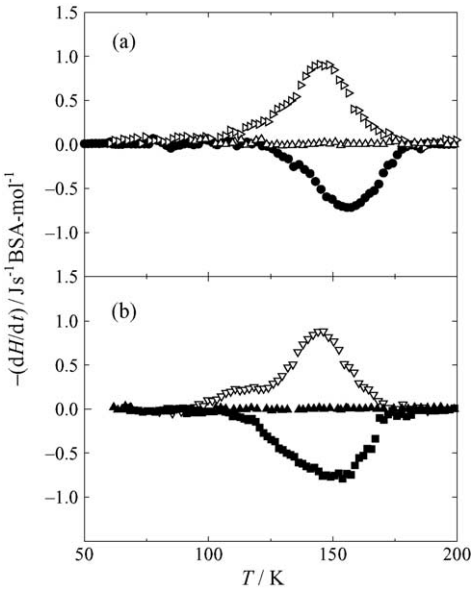

Fig. 4. Spontaneous enthalpy relaxation rates for anhydrous and 1.85% (w/w) hydrous BSA before (a) and after (b) the annealing at 400 K: △ (the third series of measurements), ▲ (fourth), anhydrous BSA cooled rapidly at 10 K min^{-1}; ▷ (third), ▽ (fifth), hydrous BSA cooled rapidly at 10 K min^{-1}; ● (second), ■ (sixth), hydrous BSA cooled slowly at 20 mK min^{-1}.

3.2. Glass transition around 150 K

Fig. 4(a) and (b) show the rates of spontaneous enthalpy relaxation observed around 150 K for the hydrous samples before and after the 400 K annealing, respectively. The data for the anhydrous BSA are also plotted for comparison. Spontaneous heat evolution/absorption effect is found definitely for the hydrous BSA: in the sample cooled rapidly at 10 K min^{-1}, an exothermic effect starts to appear at around 110 K, shows its maximum at around 145 K, and disappears at around 180 K, as shown with open triangles ▷ and ▽. In the sample cooled slowly at 20 m K min^{-1}, on the other hand, an endothermic effect starts to appear at around 120 K, shows its maximum at around 155 K, and disappears at around 180 K, as shown with closed circles and squares. The annealing at 400 K brought no remarkable change in the spontaneous enthalpy relaxation effect. Such cooling rate dependences of the spontaneous enthalpy relaxation effect indicate the presence of a glass transition. The glass transition temperature (T_g) where the relaxation time (τ) becomes 1 ks was determined to be 155 K according to the empirical relation that the endothermic relaxation rate for the slowly cooled sample shows its maximum at $\tau = 1$ ks [17,18]. The corresponding enthalpy relaxation effect was not observed for the anhydrous BSA at all, as seen from the plots with triangles △ and ▲.

In view of the fact that the spontaneous enthalpy relaxation was not detected in the anhydrous sample both before and after the 400 K annealing treatment, the glass transition is interpreted as attributed to the freezing-in of the rearrangement motion of water molecules. This interpretation is consistent with the common view [8,9] that the BSA and generally proteins without water or with a small amount of water are immobile at room temperature and the glass transition due to their rearrangement motion is expected to take place above room temperature.

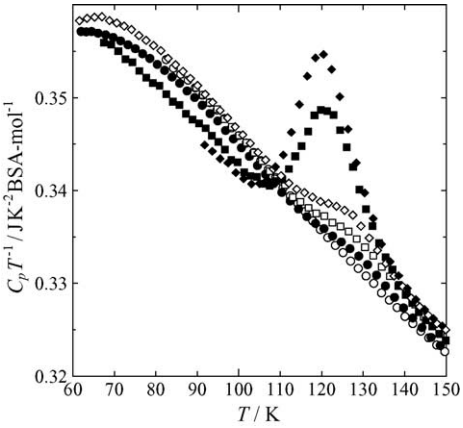

Fig. 5. Encraties (C_p/T) of 1.85% (w/w) hydrous BSA before and after the annealing at 400 K in 60–150 K: ◯, the first series of measurements; ●, second; □, fourth; ■, sixth; ◊, seventh; ♦, eighth.

3.3. Thermal anomaly around 120 K

Anomalous behavior of heat capacities was found for the hydrous sample in a range 80–140 K, while not for the anhydrous one. Fig. 5 shows the results as a plot of encraty versus temperature for clarity. The measurements of the first series were carried out in 80–280 K for the as-purchased sample. The obtained results, which are plotted with open circles, show rather smooth temperature dependence without remarkable anomaly. The results of the second series, plotted with closed circles, show a small hump in a range 120–150 K with its peak at 130 K, while giving smaller values in a range 80–115 K as compared with those of the first series. The measurements of the fourth through sixth series were carried out after the sample had experienced once the annealing at 400 K. The results of the fourth and sixth series are plotted with open and closed squares, respectively. The results of fourth series show only a small hump with its peak at 130 K. Those of the sixth series exhibit a large hump with its rather sharp peak at 122 K, while giving also a large decrease in the values in 80–110 K. The measurements of the seventh and eighth series were carried out after the second annealing treatment at 400 K, and the results are plotted with open and closed diamond, respectively. The hump becomes rather small with its peak at 127 K in the seventh series as compared with that in the sixth. The results of the eighth series, on the other hand, show the largest effect in the hump with its peak at 120 K. The decrease in the heat capacities in 80–110 K is largest as well.

The 1.85% (w/w) BSA showed an unexpected anomaly, as described above, that the heat capacities above 115 K increase and exhibit a hump around 120 K while its peak temperature moves from 130 to 120 K as the magnitude of the peak in-

creases, and that the heat capacities below 115 K decrease in comparison with the values in the case without anomaly. This feature of the anomaly indicates that it is recognized as potentially originating from a kind of phase transition. The anomaly is hardly detected in the sample immediately after the preparation at room temperature and, even after the appearance, tends to disappear through the annealing at 400 K. On the other hand, it has tendency to appear and increase while the hydrous sample is kept in the temperature range between 80 and 300 K.

As well known, the rearrangement motion of BSA molecules is frozen even at room temperature and, in fact, no anomaly can be found in the anhydrous sample. The anomaly is thus understood as ascribed to the motion of water molecules. The hydrogen-bond network formed by water molecules naturally develops with decreasing temperature based on the enthalpic request and breaks with increasing temperature based on the entropic request. This understanding is consistent with the facts that the anomaly was realized through keeping the sample at low temperatures in between 80 and 300 K and removed through annealing at 400 K. Since the diffusion process of water molecules and the development of the hydrogen-bond network are frozen in principle below 155 K by the glass transition found as due to the freezing of the motion of water molecules, it is considered that only the ordering/disordering of hydrogen-atom positions within the network can be a candidate for the degree of freedom to produce the anomaly around 120 K. Such an anomaly has not been reported, however, to occur around 120 K for bulk water or ice. The anomaly around 120 K would be therefore caused only in a situation where a certain special hydrogen-bond network structure was constructed in association with formation of the hydrogen bonds between water molecules and parts of BSA molecules and where a small system of a particular size of hydrogen-bond network happened to be formed there. Similar situation is potentially realized in the BSA–water system with different moisture content when water molecules are introduced into anhydrous BSA contrary to the present case of removing water from the solution and the same small system of the network is formed within the BSA. The experiment for the confirmation is now under progress in our group.

4. Conclusion

Thermal properties of a dried globular protein BSA containing only slight moisture were studied in detail by an adiabatic calorimetry at low temperatures. Two notable phenomena were found as caused by the water left a little inside of BSA; a glass transition at 155 K and an anomalous behavior of heat capacities in 60–140 K. This finding provides new knowledge on the water dynamics in the protein–water system and the ordering/disordering process of water molecules of a small aggregate.

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