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Heat capacity and thermodynamic characteristics of denaturation and glass transition of hydrated and anhydrous proteins

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Abstract

Calorimetric measurements of absolute heat capacity have been performed for hydrated ^{11}S-globulin ($0 < C_{H_2O} < 25\%$) and for lysozyme in a concentrated solution, both in the native and denatured states. The denaturation process is observed in hydrated and completely anhydrous proteins; it is accompanied by the appearance of heat capacity increment ($\Delta_N^D C_p$), as is the case for protein solutions. It has been shown that, depending on the temperature and water content, the hydrated denatured proteins can be in a highly elastic or glassy states. Glass transition is also observed in hydrated native proteins. It is found that the denaturation increment $\Delta_N^D C_p$ in native protein, like the increment ΔC_p in denatured protein in glass transition at low water contents, is due to additional degrees of freedom of thermal motion in the protein globule. In contrast to the conventional notion, comparison of absolute C_p values for hydrated denatured proteins with the C_p values for denatured proteins in solution has indicated a dominant contribution of the globule thermal motion to the denaturation increment of protein heat capacity in solutions. The concentration dependence of denaturing heat absorption (temperature at its maximum, T_D, and thermal effect, ΔQ_D) and that of glass transition temperature, T_g, for ^{11}S-globulin have been studied in a wide range of water contents. General polymeric and specific protein features of these dependencies are discussed.

Keywords: Calorimetry; Proteins; Denaturation; Heat capacity; Denaturation increment of heat capacity; Vitrification

1. Introduction

The minimum water content necessary for the preservation of the native protein structure does not exceed 25–30% of their total mass [1,2]. Further increase in the water content does not change essentially the ordering of the native structure. On the contrary, a water content below the indicated value changes the native structure, as indicated by the decline in the enzymatic activity of proteins, by decreased thermal effect of denaturation on heating, or by destruction of the crystalline protein structure [2–6]. However, the presence of even small amounts of water proves to be sufficient for the appearance of a denaturation peak of thermal absorption in the heating thermogram of the native protein, as is the case for solution. The denaturation peak is absent on

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repeated heating (irreversible denaturation), but a 'step' characteristic for glass transition [7–10] appears at certain temperatures like in all synthetic polymers.

We have recently shown that structural changes occurring on native protein dehydration give rise to a disordering phase which, like the amorphous phase in semicrystalline polymers, can pass to the glass on cooling [11,12]. The main chain of the amorphous polymer loses its flexibility in the glass state, resulting in the disappearance of the translational degrees of freedom.

The interest in the study of the protein glass transition in the systems of low moisture is growing due to their importance in food science and technology. The fact is that, at a low water contents, the glass transition of denatured and native proteins, like other biological macromolecules, is observed in the temperature range of technological processing and storage of foodstuffs. It is this circumstance that has led to the rapid development of physicochemical, in particular, calorimetric studies of vitrification in biopolymer–water systems and the establishment of the plasticizing role of water, an integral component of foodstuffs [13–15]. Numerous experiments have shown that the protein vitrification temperature drops as the amount of protein-bound water increases. There has been a suggestion that the plasticizing effect of water is linked with the replacement of interchain protein–protein bonds by protein–water bonds increasing the segmental (translational) mobility in the protein molecule [16].

Most studies on the structure, stability and thermodynamic properties of proteins have been done for dilute solutions, and, in part, for moderately concentrated solutions of natural polymers. In the presence of 'free' water, the glass transition of the protein backbone cannot be observed, since under such conditions the glass transition temperature, T_g, is always below the water freezing point. On freezing, water freezes the highly elastic state of the protein. By highly elastic state, we mean the amorphous polymer state in which torsional vibrations in the chain backbone occur, providing flexibility of the chain segments. The set of possible conformations of the protein chain depends on the cooling kinetics and does not change with time. On subsequent equilibrium heating ($v_{heat} \Rightarrow 0$), the set of conformations characteristic for highly elastic state for any given temperature immediately appears in the chains at $T > 0$°C. A highly elastic denatured protein is in equilibrium state. In contrast, the glassy state in the absence of free water, or the state frozen in the free water matrix are metastable ones.

Calorimetric investigations of Privalov et al. [17] have shown that the heat capacity of the heat-denatured protein in dilute solution is close to that of a completely unfolded random coil and, therefore, can serve as a baseline for absolute heat capacity readings for other states of the protein. For the calculations, it is sufficient to measure the heat capacity difference, ΔC_p, between the denatured protein and the protein of interest, using a differential scanning calorimeter. This imposes stringent requirements on the accuracy of heat capacity measurements for the denatured protein.

Unlike dilute protein solutions, hydrated proteins can undergo the processes of softening and denaturation in the temperature range of -50–200°C, as is shown by the temperature dependence of heat capacity of the native protein. The softening process of denatured hydrated protein on its heating, which represents the transition from glass to highly elastic state, is revealed at the temperature dependence of C_p in the form of a well-pronounced step.

Calorimetric studies of low humidity proteins besides their practical significance mentioned above are also important as fundamental studies of the peculiarities of polymer structure behavior in the presence of solvent. In this work absolute values of heat capacities for two denatured proteins, ^{11}S-globulin and lysozyme, at low humidity and in solution state have been determined with sufficient accuracy. The comparison of absolute values of protein heat capacities in denatured states in the presence, as well as, in the absence of free water, and even water, in general, provide a unique experimental possibility to estimate quantitatively the contribution of hydrophobic interactions into the heat capacity of denatured protein in solution. Carried out in this work, the comparison of temperature dependencies of heat capacity values for proteins with different water content and concentration dependencies of vitrification and denaturation parameters (T_g and T_D, ΔQ_D) can be of help in understanding which protein properties are mainly controlled by their backbone struc-

ture, and which ones are related to the tertiary structure of the globule, being unique for each protein and conditioned by the presence of water.

2. Materials and method

2.1. Heat capacity

Absolute heat capacity has been studied as a function of temperature in the range −50–200°C for globular protein ^{11}S-globulin (hereafter denoted as legumin). Legumin separation from *Vicia faba* bean seeds was carried out at the Institute of Elemental Organic Compounds, Russian Academy of Sciences, Russia. Its purification from ^{7}S-globulin was performed by isoelectric sedimentation in salt solutions [18]. The protein obtained contained 95% legumin and 5% of its dimer. A homogeneous solution of this protein with concentration $C = 55\%$ in 0.6 mol/dm^3 NaCl, pH = 4.7 was obtained at the same institute using the phase stratification method for moderately concentrated protein solutions. Legumin thermal stability and the denaturation increment of heat capacity in dilute solution had been determined earlier [19]. Lysozyme was a commercial specimen (Sigma).

The calorimetric accuracy of absolute heat capacity varies with the amount of the substance measured. Steel-sealed ampoules used for the measurements can hold up to 100 mg of dry protein. We always tried to achieve the maximum filling of the ampoules.

Protein samples of definite water contents were prepared by drying a homogeneous 55% aqueous solution. The water content was varied with the target of the experiment either by additional wetting of the sample in water vapour, or by vacuum drying at room temperature, so that the protein humidity was varied from 0 to 25%. The water content was determined from the dry weight after vacuum treatment at 120°C for 1 h. To obtain a uniform water content, a hydrated sealed sample was allowed to stay for 7 days before the measurement. To prepare anhydrous protein, vacuum drying was carried out at $T = 120$°C to a constant weight. The heat capacity was measured with a Setaram DSC-111 differential scanning calorimeter equipped with an IN-50 computer. The scanning rate was 5 K/min.

For the calibration, we used Al_2O_3, a standard substance for calorimetric investigation. The sample weights and the temperature range of heat capacity measurements for Al_2O_3 and proteins were identical. Our results on Al_2O_3 specific heat are given in Fig. 1, along with the data obtained by Furukawa et al. [20] from adiabatic calorimetry often used as standards in calorimetric studies. Our results coincide with the previous data [20] within 1% for $T > 0$°C and within 3% for $T < 0$°C. To check the correction for the temperature delay at the heating rate of 5 K/min, introduced by the computer, we used data on the melting point of In. For that purpose, a piece of high-purity In (In 99.999%) was placed into an ampoule containing Al_2O_3.

In the calculations of absolute heat capacity from experimental data for hydrated proteins, we assumed C_p of hydrated protein to be the sum of the respective values for the protein, 'bound' water and small amount of NaCl. The data by Hutchins et al. [21] were used for the heat capacity of 'bound' water at $T < 20$°C. C_p of 'bound' water at $T > 20$°C was assumed to be the same as that of free water. The correctness of this assumption is confirmed by the heat capacity values of water, which we obtained by extrapolation of the heat capacity–concentration plot for the solution to zero protein concentration at different temperatures. All extrapolated plots intersected at the point $C_p = (4.3 \pm 0.1)$ J g^{-1} K^{-1}.

No water evaporation into the atmosphere took place during heat capacity measurements. However, evaporation inside the sealed ampoule is to occur during the temperature scanning. Large water weights

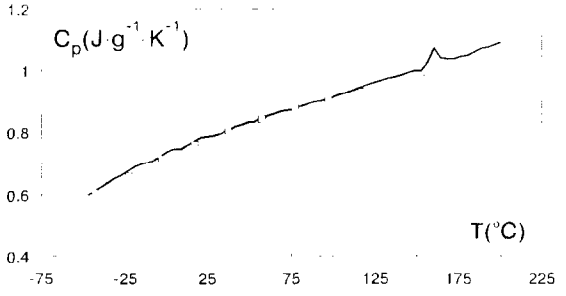

Fig. 1. Temperature dependence of specific heat for Al_2O_3. The solid line corresponds to the values obtained in this work; (□) represents the data of Furukawa et al. [20]. The maximum at 156°C corresponds to melting of the temperature marker, In.

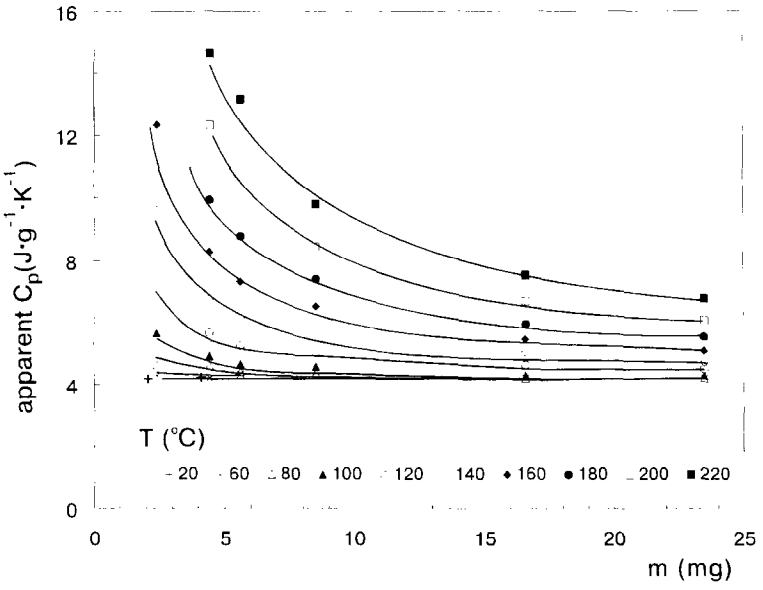

Fig. 2. Apparent specific heat of water as a function of water mass in sealed ampoule at different temperatures.

are usually used in such measurements in a sealed ampoule; therefore, the contribution of evaporation heat to specific heat of water is usually negligible. One should expect that the contribution of the evaporation heat of water to its specific heat may increase as the mass of water in the ampoule decreases.

A series of measurements has been performed to estimate the error due to evaporation in the heat capacity of proteins with low water contents. The mass of water in these measurements coincided with that in the protein samples (23.5; 16.6; 8.5; 5.6; 4.6; and 2.4 mg H_2O). To bring the evaporation of small amount of free water (initially a water drop) close to that of water bound to protein with a larger evaporation surface, we heated the free water up to 200°C and then cooled it down to $T = 0$°C prior to the heat capacity measurement. Then the water evaporated on heating condensed onto the internal ampoule surface. The evaporation surface on subsequent heating was increased considerably, which brought the evaporation of free water closer to that of bound water. These results are given in Fig. 2 showing that, for small weights of water, its evaporation distorts the true heat capacity of the water in such a way that its apparent heat capacity is the larger; the smaller is the water mass; and the higher is the temperature. We used the water heat capacities, including the evaporation inside the ampoule, in the calculations of protein heat capacities at $T > 0$°C. The values obtained for protein heat capacities are somewhat lower than C_p values presented in our previous papers. The distinction manifests itself at $T > 120$°C, making up 4% at 120°C and reaching 10% at $T = 200$°C.

2.2. Thermal effect of denaturation

The denaturation heat and temperature in a reversible process are thermodynamic characteristics of the protein, whereas the thermal effect of denaturation and T_{max} in an irreversible process, similar in its manifestation in the thermograms, can be used only as comparative denaturation characteristics, providing the heating rate is known. The endothermic maximum of heat absorption in an irreversible process represents the superposition of either aggregation heat release, or of chemical processes, proceeding with heat release upon denaturation. The way of finding the baseline in these conditions and, hence, the way of calculating the denaturation thermal effect for definite heating rates were given in detail in our review [22].

2.3. Temperature

The temperature of maximum denaturation heat absorption was used as the denaturation temperature,

T_D. The glass transition temperature, T_g, was found as a temperature, at which the second derivative $d^2C_p(T)/dT^2$ changes its sign at the sigmoidal increase of the protein heat capacity.

3. Results and discussion

3.1. Plasticizing role of water and protein thermostabilization on dehydration

Fig. 3 presents the thermograms of hydrated legumin in the native (1) and denatured (2) states. The peaks in curve 1 are the results from denaturation of the native structure remnants. Thermal denaturation in hydrated legumin is always irreversible. For this reason, the second heating of the sample characterizes its denatured state. Consider the thermograms of denatured legumin sample with 6% H_2O (Fig. 3b, curve 2). The temperature range +25–+100°C containing a step of $C_p(T)$ larger than before and after

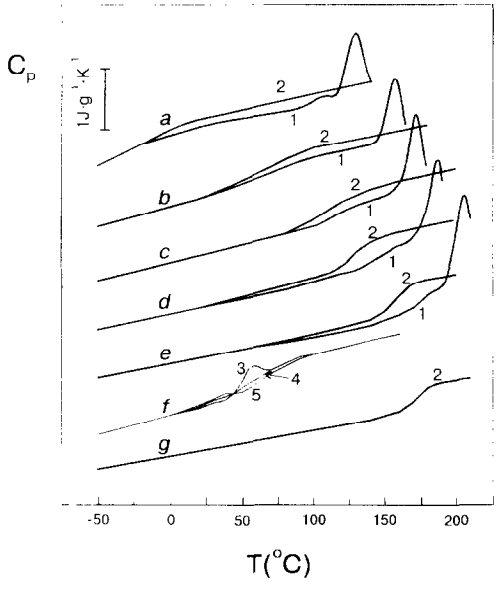

Fig. 3. Specific heat of hydrated legumin with different water contents as a function of temperature. (a) 19%; (b) 6%; (c) 4%; (d) 2.1%; (e) 0% of H_2O. (1) native protein; (2) protein denaturated by first heating. (f) represents denaturated legumin with 6% H_2O: (3) after annealing at $T = 20$°C for 70 days; (4) after cooling at 5 K/min; (5) after quenching in liquid nitrogen from 170°C. (g) represents the second heating of dehydrated legumin after denaturation in open ampoule. Details in text.

this range represents the temperature of softening/vitrification of the protein molecular backbone [11,12]. Within the softening range and at lower temperatures for the glassy state, the protein state is thermodynamically nonequilibrium and depends on the kinetics of heating, cooling and on other features related to thermal prehistory. This statement is displayed in Fig. 3 by the curves C_p (3, 4, 5), curve f.

The overall picture of the concentration dependencies for the three parameters of interest, namely for T_g, T_D and Q_D, obtained from calorimetric studies, is given in Fig. 4. In the present paper, the plot of denaturation parameters vs. concentration is discussed with the use of our data on legumin glass transition. One should note that the calorimetric studies of the concentration dependence of T_D and ΔQ_D for proteins with low water content had been performed long before glass transition was discovered in such systems. As a result, T_D was found to increase sharply with the increase of protein concentration from 70 to 100% (with water as a plasticizer), whereas ΔQ_D decreases, as a rule, under these conditions [16,23,24]. Our results have confirmed the regularities obtained earlier and revealed some new details in the plots of ΔQ_D and T_D vs. concentration at near-zero water content, unnoticed in the previous studies.

It follows from Fig. 4 that the denaturation temperature of native anhydrous legumin (determined for 5 samples) is $T_D = 206$°C, and the glass transition temperature is $T_g = 190$°C. The decrease of both temperatures occurs on protein moistening T_g decreases more rapidly than T_D does. At water content of 50%, the denaturation temperature reaches the value $T_D = 100$°C (within the accuracy 3–5°C), i.e., the value obtained for dilute solutions, and does not decrease on further increase of the water content. Therefore, T_D of legumin increases by 106°C if we go from the solution to the dry protein.

The lowest glass transition temperature we obtained experimentally for legumin is −30°C. It corresponds to the maximum possible content of bound water (25–28% of total mass of solution). The appearance of even infinitesimal amounts of free water distorts, due to its freezing, the hydrated protein thermogram, thus preventing the correct determination of T_g. As for T_g of the protein in solution, one

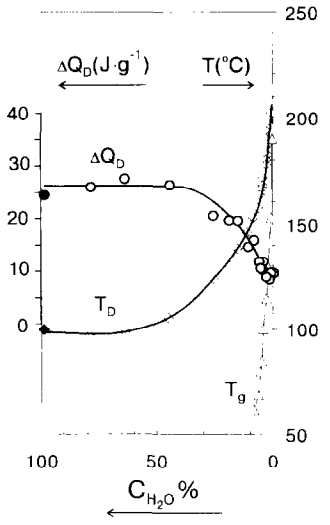

Fig. 4. Concentration dependencies of the parameters of denaturation T_D and ΔQ_D, and of the temperature of vitrification T_g of legumin.

can only say that it is much lower than $-30°C$. Since the protein solutions are dealt with at $T > 0°C$, one should keep in mind that the denatured protein and the disordered part of the native protein in solution are always in highly elastic state.

The fall in the melting temperature in the presence of a solvent has a well-justified thermodynamic explanation in polymer physics. The equality of the chemical potentials of the polymer in crystalline and liquid states, the main condition of melting in the absence and presence of solvent, appears at different temperatures because of the energetic and entropic factors of the melted polymer mixing with the solvent. The solvent lowers T_{melt}. However, as far as we know, the lowering of T_{melt} of synthetic polymers in the presence of solvent does not exceed 50°C. This effect in proteins may reach 100°C and more. As the native globule of protein does not contain water within it, the mixing effect on protein denaturation should also take place. In Fig. 5, the experimental values of T_D of legumin are presented as function of C_{H_2O}/T_D for reference to synthetic polymers [25]. The comparison of the dependencies of denaturation temperature, T_D, for protein, and T_{melt} for polymers on water concentration shows a close similarity for water contents above 20%. The discrepancy appears when 'bound' water leaves the protein surface. As this takes place, T_D for protein shows more pronounced concentration dependence. Flory and Garret [26] was the first who observed such difference in the behavior of fibrillar protein collagen. Our results for globular protein agree with Flory's results for collagen.

When the water content in proteins decreases below 25%, the destruction of the globule-ordered structure takes place, which should somewhat slow down the rise in T_D (because of $T_D \cong \Delta Q_D/\Delta S_D$). However, T_g in proteins, unlike synthetic polymers, sharply rises with the fall of water content (Fig. 4). This process is so strong as to narrow the temperature interval between T_D and T_g from 100°C to 10°C in the vicinity of zero moisture. One can assume that some critical level of segmental mobility should be reached in disordered protein phase softened after vitrification, for the protein denaturation to occur. The temperature at which this occurs, namely T_D, should, first, be higher than vitrification temperature always; second, it must exceed T_g by the value decreasing with a rise of temperature due to the temperature dependence of segment kinetic energy. The results confirm these assumptions (Fig. 4). Thus, for low water contents (< 20%), the additional sharp rise in T_D in proteins in comparison with that in synthetic polymers can be a consequence of more pronounced dependence of T_g on water content.

Next, let us consider a dependence of another

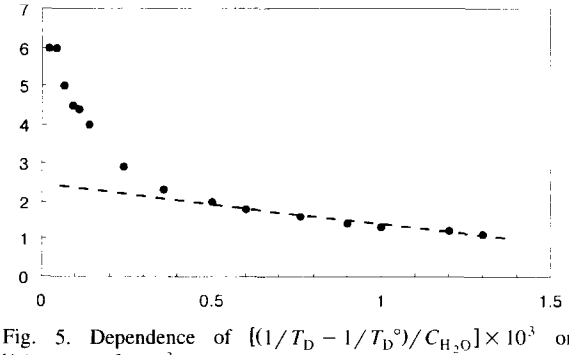

Fig. 5. Dependence of $[(1/T_D - 1/T_D^\circ)/C_{H_2O}] \times 10^3$ on $[(C_{H_2O})/T_D] \times 10^3$ for legumin in presence of water. T_D° is the denaturation temperature of dehydrated protein. The broken line indicates the behavior expected for synthetic polymers. Details in text.

denaturation parameter ΔQ_D on the concentration. We noted above that proteins, even those in completely anhydrous state, reveal the denaturation maximum at the thermogram (Fig. 3, curve a1). Investigation of 17 legumin samples with water content from 6 to 0% has shown that the thermal effect of denaturation reaching a value of (8.5 ± 2) J g^{-1} ceases to diminish when the water content tends to zero. This result was unexpected for us. Extrapolation of ΔQ_D to zero for dry protein performed in previous works seemed to be justified: the more the water stabilizing the native structure is removed from the globule surface, the greater distortions appear in the native structure. In the limit of complete dehydration, this structure must be disrupted, i.e., $\Delta Q_D \to 0$.

Which processes occur in protein at the final stage of its dehydration, that lead to the stabilization of the native structure remnants? The answer, as in the case of the rise in temperature stability, in our view, is the same—a sharp rise in T_g of the disordered phase for low humidities. Heat denaturation of protein goes on in a cooperative manner. On the contrary, denaturation at dehydration occurs gradually and may be stopped at any stage. During the process of native protein dehydration, the increase of vitrification temperature of disordered phase takes place beside the denaturation of ordered (tertiary) phase. Vitrification temperature of completely dehydrated protein is usually higher than the temperature of the beginning of its thermal decomposition. Therefore, the temperature of dehydration should be chosen in such a way as to prevent protein decomposition and to provide the highest possible dehydration rate at the same time. In our experiments, the pumping temperature $T_p \cong 100$–120°C was the optimal temperature for dehydration.

As long as the protein humidity was higher than 8% H_2O during the process of protein pumping, the vitrification temperature of the protein was lower than the pumping temperature $T_p = 120$°C. In the dehydrated part of the globule of such protein, the structure equilibrium characteristic for highly elastic state was established. However, as soon as the humidity reached 8% H_2O, T_g of such protein became higher than T_p and abstraction of water molecules from the surface of the globule occurred under conditions where parts of the globule freed from water appeared in glass state. For a similar reason, the parts of the globule would be forced to conserve the chain structure that had placed in the presence of water.

The disruption of glass-ordered structure occurs upon heating of protein with mentioned humidity ($\leq 8\%$ H_2O) from 120° to 220°C at the rate of 5°/min. The contribution of the structure in the globule heat capacity should be the same for various humidities from 8 to 0% H_2O. It is precisely this situation that is exhibited in Fig. 4, curve for ΔQ_D.

Thus, the vitrification of disordered phase, not the water bounded with protein, exerts a stabilizing effect on the remnants of protein native structure at the last stages of protein dehydration.

In conclusion, we would note an important peculiarity of thermograms for low-humidity proteins. As Fig. 3 shows, T_g for native protein at low humidity is always higher than T_g of protein denatured under the first heating. We have some preliminary findings from which it follows that at high temperature of denaturation in protein, a chemical reaction probably takes place with the release of water (for example, condensation of the aminocarboxylic groups). The water released wets the denatured sample, thus decreasing its T_g. To obtain the coinciding T_g values in native and denatured states of anhydrous samples, protein must be denatured either in solution, i.e., at low temperatures, or in open ampouls to provide the evaporation of the water released. The respective experiments have shown that the values of T_g for denatured and native proteins were brought much closer together in this case, Fig. 3, curve g.

3.2. *Absolute heat capacity for legumin*

Consider in succession the absolute heat capacity for legumin in denatured and native states.

3.2.1. C_p *for denatured legumin*

3.2.1.1. Highly elastic state. Heat capacities of denatured legumin in highly elastic state (Fig. 6) were calculated from the experimental C_p data for this protein with different water contents, under the assumption that heat capacity of hydrated protein is the sum of the respective values for protein and water. The C_p data for proteins with water content in the

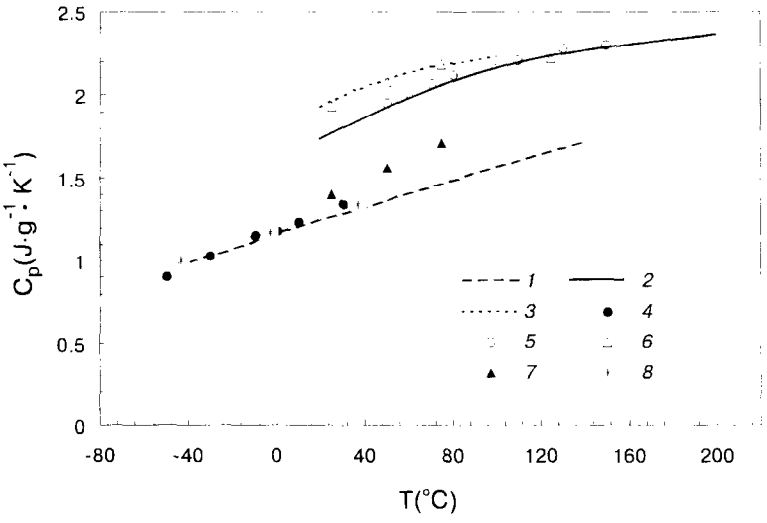

Fig. 6. Specific heat of legumin and lysozyme in glass, highly elastic and solution states. Legumin: (1) glass state; (2) highly elastic state (values were averaged over all moisture contents for the same temperature); (3) concentrated solution of denaturated legumin. Lysozyme: (4) glass state; (5) highly elastic state; (6) dilute solution of denaturated lysozyme [30]; (7) dilute solution of native lysozyme [30]; (8) glass state of dehydrated chymotrypsinogen [21].

range 0–44.9% were used for the calculation. All the features of water heat capacity, considered in the course of these calculations, were discussed in detail in Section 2.

The results obtained confirm the assumption that water in proteins plays the role of plasticizer displacing the region of the transition from glass to highly elastic state toward lower temperatures [14]. Methodical limitations from low-temperature side prevent the observation of the whole vitrification range for water contents higher than 11.4%. On further increasing of the water content to 25.6%, the ultimate transition into glass occurs at temperatures below −30°C. Only the end of the softening process and the transition of the sample into highly elastic state is recorded on heating in the thermograms of proteins with such water content. At water contents above 25.6% the free water appears in the samples. Its melting at 0°C masks completely the variations of C_p associated with glass transition in the thermogram.

The interval between the nearest values of water content was chosen in such a way as to have the overlapping temperature ranges for C_p values of the proteins in a highly elastic state. The scatter of C_p values for the protein with different water content at the definite temperature did not exceed 2% in the temperature range +20–+100°C. The C_p temperature dependence for denatured legumin in highly elastic state is $C_p = A \cdot T^{1/n}$ ($n > 1$). The linearization of this plot over short temperature intervals shows that at low temperatures the change of T by 1° increases C_p by 0.005 J g^{-1} K^{-1} and for $T >$ 120°C, by 0.002 J g^{-1} K^{-1}, Fig. 6, curve 2.

3.2.1.2. Glass state. C_p values for legumin in glass state were also calculated from the thermograms of respective hydrated proteins with different contents of bound water. The calculations have shown that the values of C_p obtained agree with C_p anhydrous protein in glass state within 2% in the whole temperature range, where the data for anhydrous protein were available, i.e., −50–+140°C. In this connection we presented on Fig. 6 and in Table 1 the C_p values of completely anhydrous protein averaged over 8 samples, for C_p legumin in glass state. In this case the scatter of C_p values did not exceed 3% for $T < 0$°C and 1% for $T > 0$°C. The values of C_p for legumin in glass state coincided with high accuracy with respective values for anhydrated chymotrypsinogen, obtained by Hutchins et al. [21].

Table 1
Temperature dependence of specific heat absolute values of legumin and lysozyme in the presence of free and bound water

Hydrated legumin and lysozyme		
Glass state		Highly elastic state
T (°C)	C_p^N (J g^{-1} K^{-1})	C_p^D (J g^{-1} K^{-1})
−40	0.99	
−20	1.07	
0	1.17	
20	1.26	1.75
40	1.32	1.87
60	1.41	1.96
80	1.49	2.07
100	1.57	2.18
120	1.65	2.22
140	1.72	2.25
160		2.28
180		2.32
200		2.36

Solution of lysozyme		
Denaturated lysozyme		
Concentrated solution		Dilute solution[a]
T (°C)	C_p^D (J g^{-1} K^{-1})	C_p^D (J g^{-1} K^{-1})
25	1.96	1.93
50	2.08	2.07
75	2.17	2.18
100	2.23	2.21
125	2.30	2.21
Native lysozyme		
Concentrated solution		Dilute solution[a]
T (°C)	C_p^N (J g^{-1} K^{-1})	C_p^N (J g^{-1} K^{-1})
25	1.53	1.40
50	1.66	1.56
75	1.79	1.71

[a]Data from Makhatadze [30].

Heat capacity of denatured legumin in glass state increases linearly with temperature within the range −40–+140°C with the gradient 0.004 J g^{-1} K^{-2}, Fig. 6, curve 1.

3.2.2. C_p for native legumin

Consider the variations of its heat capacity in the course of two successive heatings in the temperature range −50–+200°C with an example of legumin containing 4% of water, Fig. 3, curve c. These C_p variations are caused by protein conformations only. The curve of first heating (1) characterizes the temperature dependence of C_p for native protein, the one of the second heating (2) characterizes $C_p(T)$ for denatured protein due to irreversible character of denaturation. In the glass state (−50–+80°C) the values of C_p for native and denatured states coincide and grow linearly with temperature. Above 60°C, both samples show more pronounced increase of C_p, although different for both states of the protein. The C_p value of native protein after its softening is lower than C_p of denatured protein. In denatured sample in the presence of water, the near-linear $C_p(T)$ dependence with the slope, lower than that in the temperature region of glass state, occurs after the completion of the softening process. In native sample, the partial softening is completed by the sharp endothermic maximum (T = 171.5°C), characterizing the denaturation of native structure remnants. In this connection, it seems more correct to define the state of the protein with the content of bound water below 25%, as a semi-native one, by analogy with semicrystalline synthetic polymers. The difference in heat capacities of denatured and native states of the protein, after the curves $C_p(T)$ diverge, on the sample leaving the glass state, is that $C_p(T)$ for the former increases in one jump on protein softening, while for the latter it increases in two jumps—on the softening and denaturation. After denaturation, $C_p(T)$ for native and denatured proteins should coincide, as being related to the same state.

It should be noted, that denaturation in hydrated protein, as well as that in dilute solutions, is accompanied by an increment of heat capacity. The value of this increment can be defined as the difference between heat capacities of denatured and semi-native proteins inside the denaturation range. At high content of bound water, the softening process in semi-native protein reaches saturation long before denaturation. In this case, the heat capacity difference of two parallel plots for denatured and semi-native proteins gives a clear idea of the value of heat capacity increment on denaturation (Fig. 3, curve a).

Softening (or the reverse process of vitrification) is a relaxation process in which a part of vibrational degrees of freedom of the polymer backbone is transformed into hindered rotation upon heating. The latter is the trigger mechanism, initiating morphological changes in the structure of amorphous polymers as temperature increases [27]. As a result, a new type of motion appears in polymer, i.e., translation of individual chain segments in the bulk. As this takes place, the polymer is transformed into a highly elas-

tic state. It is the appearance of new, additional degrees of freedom in the temperature range of softening, that results in such a large increase in polymer heat capacity. Since the heat capacity values of the native protein after denaturation and of the denatured one after softening coincide, the following conclusion seems to be quite logical: the heat capacity jump on denaturation in hydrated native protein is also connected with the excitation of additional degrees of freedom, which are liberated as a result of cooperative thermal destruction of the elements of protein tertiary structure [11,12].

3.3. Effect of free water upon the appearance of the denaturation increment of heat capacity and its value

The establishment of the existence of heat capacity increment upon denaturation for protein in solution, as well as the detection of the temperature dependence of denaturation enthalpy, is one of the most important achievements of protein experimental physics during the last 20 yrs. Both peculiarities play the dominating role in the construction of protein thermodynamics. Besides, the denaturation increment of C_p is used as the basis for hydrophobic concepts of the process of protein globule heat unfolding, and is also a necessary component in the justification of the cold protein denaturation. In the recent years, the heat capacity increment upon denaturation has been the subject of repeated studies to obtain its value and temperature dependence with greater accuracy [17,28,29]. At present, the idea of $\Delta^D_N C_p$ hydrophobic origin is generally accepted. Hydrophobic hydrocarbon radicals inside the native globule come into contact with water as a result of denaturation. Such contact, being energetically unfavourable, results in the orientation of water molecules and the formation of cluster. Further, upon heating of such a solution, the additional heat absorption is required for gradual destruction of these clusters. The latter results in heat capacity increase as a result of denaturation, i.e., in the appearance of $\Delta^D_N C_p$. The necessary element of these ideas is the presence of free water in the system water–protein.

We have shown above that the increment of heat capacity upon denaturation exists also when free water is lacking. Its value correlates with the heat capacity increment upon the processes of softening/vitrification of denatured protein, and can be described in the framework of the theories for these processes. In this case, the increment is the manifestation of the properties inherent to the protein itself, but not water. This is confirmed by the existence of $\Delta^D_N C_p$ in completely anhydrous native protein. The bound water affects only the denaturation temperature. It is quite natural to suppose that heat capacity increase of this origin should also contribute to the heat capacity with the free water present. The question is: which mechanism, either hydrophobic or translational, dominates the appearance of the heat capacity increment upon denaturation of the protein in solution?

To answer the question, let us compare the heat capacity values of the protein in denatured state for the cases when free water is present or lacking. Hydrophobic effect can occur only in the former case. The increase of protein's C_p after denaturation in solution as compared to C_p of hydrated denatured protein in high elastic state should characterize the hydrophobic contribution to protein heat capacity under its unfolding in solution.

In this research, besides legumin in hydrated state, we have studied lysozyme with 7% and 15.3% H_2O and in concentrated solution ($C^{prot} = 25\%$ and $C^{prot} = 37\%$). Heat capacities of hydrated lysozyme in denatured state coincided with C_p values for denatured legumin in both highly-elastic and glass states, Fig. 6. Lysozyme has been chosen as C_p values for this protein in dilute solution have recently been obtained for both denatured and native states [30]. C_p values for lysozyme in concentrated and dilute solutions are presented in Table 1. One can see from the table that C_p values for denatured lysozyme, obtained in our experiments, coincide with respective values for dilute solutions, as presented in Ref. [30]. This means that the value of heat capacity of denatured protein in solution does not depend on its concentration.

On the other hand, C_p values of lysozyme in solution are somewhat higher than the values of C_p for hydrated denatured protein in highly elastic state in temperature range $+20 - +100$°C. At $T = 20$°C, the value of C_p of the protein in solution is 10% greater than C_p value for protein in hydrated state.

This difference decreases with the increase of temperature, disappearing completely at $T = 100°C$. Heat capacity increment upon denaturation for lysozyme in dilute solution $\Delta_N^D C_p = C_p^D - C_p^N = 0.564$ J g^{-1} K^{-1} at 20°C (see Table 1), C_p of protein in solution exceeding the respective value for hydrated protein by only 0.146 J g^{-1} K^{-1}, i.e., by 26%. Therefore, the free water and the hydrophobic effect associated with it are responsible only for 26% of denaturation heat capacity increment of protein in solution. The remaining 74% of increment are related to the appearance of new degrees of freedom as a consequence of denaturation, namely to the translation motion of chain segments. The conclusion drawn disagrees with presently accepted concept on the dominating role of hydrophobic effect in the increase of heat capacity of proteins in the course of denaturation in solution.

Nevertheless denying the importance of hydrophobic effect in the physics of folding/unfolding processes of globular protein, we merely state that segmental mobility following denaturation of protein defines, to a higher extent, the value of denaturation heat capacity increment of protein in solution than do the hydrophobic effect and allied melting of oriented water clusters.

Reexamining Table 1 should call attention that C_p values of native protein in concentrated solution, unlike C_p of denatured protein, are higher than respective values in dilute solution. This fact seems to be quite natural and related to the different degrees of perfection of the globule tertiary structure. The globule of protein in dilute solution should have the highest ordering degree and, therefore, the lower heat capacity.

The important result of the present work is the establishment of relationship between biophysical (denaturation) and polymeric (glass transition) properties of globular proteins. As our experiments have shown, the individual properties of proteins are controlled to a large extent only by the fact that they belong to a broad family of linear polymers. This result appears to be nontrivial, since each protein has its own unique native structure defining its exceptional role in the processes of vital biological activity.

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