Section 1. Materials Science

https://doi.org/10.29013/ESR-22-5.6-3-10

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PULSED DIFFERENTIAL CALORIMETRY OF THE HEAT CAPACITY JUMP AT DENATURATION OF COLLAGEN TYPE I OF RAT TAIL TENDONS

Abstract. The estimation of the heat capacity jump at melting of collagen fibers having principle significance for determination of the role of hydrophobic interactions in the process of formation and stabilization of collagen fibers was performed for collagen type I of rat tail tendons in an aqueous medium and in 0.5M acetic acid. The research was performed by using the unique high-precision pulsed differential scanning calorimeter (PDSC) designed by the authors. The device provides a

measurement of heat capacity in the pulsed mode under the thermodynamically equilibrium conditions, in contrast to usual differential scanning calorimeters (DSC) performing the measurements in the continuous heating mode. The measurements carried out in the pulsed mode allow the exact determination of heat capacity before and after denaturation transition. The experiments showed that magnitude of the heat capacity jump in the aqueous medium is twice as large as the value of the jump in 0.5M acetic acid. The melting temperature in the aqueous medium was by 22 °C higher than in acetic acid and, the melting enthalpy in the aqueous medium was more too. The obtained results are discussed in the light of the data available in the literature regarding this problem.

Keywords: Collagen fibers melting, Pulsed differential scanning calorimetry (PDSC), Heat capacity jump at denaturation of collagen, Hydrophobic interactions.

Introduction

Collagen is the basic protein of the connective tissue in living organisms. It attends support and protective functions, and plays an important role in the processes of differentiation and growth of the organism, and its adaptation to the environment in the process of evolution. The functional diversity of collagen has determined the interest to its structure and biology among researchers for decades [1–8]. The problem of the formation and temperature stability is among key ones in the biology and physics of collagen. Temperature plays an essential role in the vital activity of biological systems. All catalytic processes proceeding with participation of biological Macromolecules, the temperature coefficient of which is higher than that of low molecular compounds, both in *vivo* and in *vitro* depend on the temperature. Various mechanisms of adaptation to the changes in the environmental temperature resulted in the existence of the variety of living systems and hence in the variety of biological macromolecules functioning optimally only at the physiological body temperature for homeothermic animals and at some optimal temperature for endothermic animals.

There is not observed a direct relation between the degree of the evolution of collagen and the degree the heat stability. Hence it is assumed that the phylogenetic changes in heat stability hold only adaptive significance [9]. Phylogenetic adaptation occurs at the level of the genetic apparatus and involves irreversible profound changes in biological macromolecules [10], unlike season adaptations that are short-term and of a reversible character. The collagen molecule is actually the only bio macromolecule the temperature of denaturation of which strictly follows the physiological body temperature.

The process of collagen biosynthesis results in the formation of collagen fibers, the stability temperature of which exceeds the temperature of denaturation of a triple helix in the solution by 22 °C in average [1]. Though there is some success in understanding the nature of forces responsible for the increase of fibril stability the problem has not been adequately studied yet. Some contribution to the increase of the temperature of fibril melting, as compared to the triple helix, can be made by hydrophobic interactions [2], which, as it is known, are of entropy nature.

As is known from the study of thermodynamic properties of globular proteins, for the estimation of the role of hydrophobic interactions the most efficient approach is the measurement of the heat capacity jump at the transition of protein from native state to the denaturation one [2]. In the same work, the value of the heat capacity jump with the denaturation of rat skin collagen macromolecules was assessed according to circumstantial evidence. As for fibrils there is no evidence even of that kind. Therefore, in this work the effort was made to estimate by direct precision calorimetric measurements the value of the heat capacity jump at melting of the collagen fibers of rat tile tendons in water, where collagen remains as fibrils, and in 0.5 M acetic acid, in

which collagen exists as separate triple helixes and has different degrees of stability [1] i.e. different temperatures of denaturation.

A unique pulsed differential scanning calorimeter (PDSC) created by the authors allows performing the measurements of the above-mentioned heat capacity jumps in a pulsed mode under the thermodynamically equilibrium conditions with high precision, in contrast to conventional differential scanning calorimeters (DSC) performing the measurements in the continuous heating mode under not un sufficiently equilibrium conditions. Obtained results are discussed in the light of existing representation on the properties of the triple helix and collagen fibers [11].

Rat tail tendon preparation

Tails of six week-old rats were excised from the carcasses and released from the skeleton. The fibrils were immediately washed in distilled water, and all visible contaminants were removed from the fibrils under the microscope. In order to exclude the side effects connected with the change in the amount of hydrated water and with age-related changes [12], the experiments were carried out on the fibrils from one and the same object. Prior to the experiments, the fibrils were dried in vacuum at room temperature in order to remove the remained moisture. The collagen fibrils cleaned in this way contain of 97% of pure collagen [12]. The fibril in amount of 10–20 mg was placed into a calorimetric cell, the distilled water or 0.5 M acetic acid was added and kept for a night before starting the experiment. Another cell of the differential calorimeter contained the same amount of distilled water or 0.5 M acetic acid respectively.

Calorimeter

The measurements of a heat capacity jump at melting of collagen fibers were carried out with the help of the unique, high sensitive and precise pulsed differential scanning calorimeter (PDSC) based on the original method elaborated by the authors [13–17].

At present the most sensitive calorimeters are differential scanning calorimeters (DSC) [18–20], including temperature modulated DSCs (TM DSC) [21], but DSCs have one important disadvantage: they measure heat capacities difference between sample and standard at not strongly equilibrium conditions. The reason is that in continuous heating mode, to both cells of differential container continuously is served the same power P, because of the difference in the heat capacities of the sample and standard $\Delta C = C_2 - C_1$ they are heated unequally, but soon there comes a stationary mode when cells are heated at the same rate $\frac{dT}{dt}$, due to the transition from one cell to another heat power $P = k\Delta T$, by the thermobattery between the sells, where k is the thermal conductivity of the thermobattery, and ΔT – the temperature difference between the cells. From the simple thermodynamic calculations follow, that in a stationary mode

$$\Delta T = \left(\frac{1}{2K}\right)\cdot \Delta C \cdot \left(\frac{dT}{dt}\right) \qquad (1)$$

As can be seen, the temperature difference between the cells and therefore the value of the emf of the thermobattery between the cells (signal) depends not only the value of the heat capacity difference between the cells, but the heating rate too. The higher is the heating rate, the higher is the signal. Hence, for observation small thermal effects, when the emf of thermobattery is very small, one can increase the signal and make it measurable using high speeds of heating. However, at high heating rates, temperature gradients develop in the samples and the measurements are performed under non-equilibrium conditions, because of it the registered heat capacity difference DC(T) may differ from its true value, depending on the heating rate. About similar effect was mentioned in work [22].

The above – mentioned incompatibility of high heating rates with the adequacy of measurements limits the opportunity of measuring small heat capacity differences and the accuracy of the measurement conventional differential scanning calorimeters.

PDSC allows combine high heating rates with accurate measurements. This is achieved by using the method of pulsed heating with temperature scanning. Due to the high rate of heating during the

short but powerful impulse in the pulsed mode signal is much higher with compared the continuous heating mode, at the same time the measurements were carried out in the equilibrium conditions, since at the beginning and after the end of a short pulse sample is in thermal equilibrium conditions and the heat effect under study remains undistorted [16]. Thus, using PDSC one can measure small deviation of heat capacity with high accuracy or observe such small effects, which in studies using conventional DSC can be just as well stay undetected. Therefore, PDSC exactly suitable for the task assigned in this article: precise measuring of the small jump of the heat capacity at the denaturation of collagen.

The relative error of measurements is $\delta C/C \approx 10^{-4}$, where δC is the error of measurements and C is the absolute heat capacity of the cell.

Calorimeter capable to work in a continuous heating mode too, similar to a conventional continuous heating DSCs. The rate of scanning at the continuous heating is possible to change from 6 K/h to 60 K/h.

The described calorimeter is also effective for the study of isothermal processes with high accuracy.

Experiment

The measurements of the heat capacity jump of collagen fibers at melting were made under two different conditions: in acetic acid and in water. In one of the cells of calorimetric container 20 mg of dried fiber was placed and 0.95g distilled water or 0.5 M acetic acid added. In other cell the same amount of water or 0.5 M acetic acid was poured respectively. The amount of water was controlled by analytical scales. In order to eliminate the additional errors connected with age-related changes and with other factors, all experiments were carried out on the fibrils of one and the same object.

The estimation of size of thermal capacity jump at the melting of collagen, is not a simple task, because of many factors influencing on size of this jump. Besides the above factors it is necessary to allocate kinetic character of melting of collagen which can influence this size [22]. Therefore, to exclude this artefact it is necessary to lead measurement in equilibrium conditions that was achieved in this work.

Figure 1 shows the results of investigation of denaturation of collagen in aqueous medium and in 0.5 M acetic acid. The measurements were performed in continuous heating mode at the speed of 15K/hour. It is seen that the heat absorption peaks are shifted acros the temperature by the expected value: 22 °C and are of different shape. Phase transition enthalpy calculated from these graphs is $\Delta H = 50.2\,\mathrm{Jg}^{-1}$ in 0.5 M acetic acid and $\Delta H = 60.1\,\mathrm{Jg}^{-1}$ in water respectively.

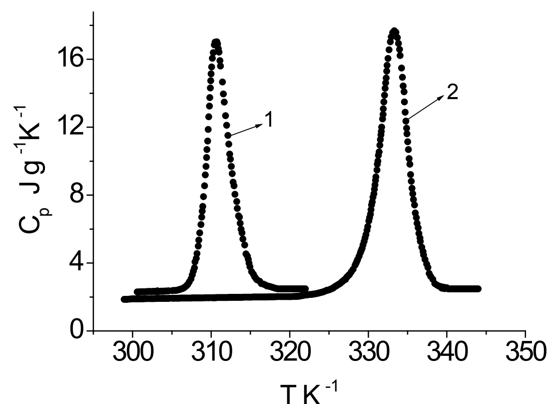

Figure 1.

Calorimetric curves of melting of collagen fibers in 0.5M acetic acid (curve 1) and water (curve 2).

Calculation the enthalpy of denaturation $\Delta H(T_d)$ occurred by numerical integration of the expression

$$\Delta H(T_d) = \int_{T_1}^{T_2} C(T)dT \qquad (2)$$

Where T_1 and T_2 are the temperature of the beginning and ending of the denaturation process, T_d is the denaturation temperature, $C(T)$ is the "heat capacity" of the collagen during the melting (Figure 1), $C(T)dT = dH(T)$ is the adsorbed heat as the temperature increases on dT – elementary area between the transition curve and the baseline. For one transition curve two basic lines are drawn: one – on the experimental points before the beginning of the transition process, below T_1, by extrapolation of which to T_d, the basic line for the temperature interval $T_1 - T_d$ was obtained and the second – on the experimental points after the

end of the transition process, above T_2, by extrapolation of which to T_d, the basic line for the temperature interval $T_d - T_2$ was obtained

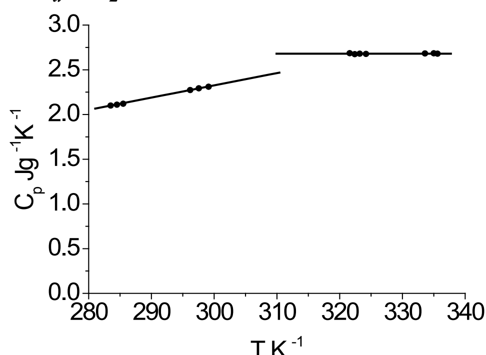

Figure 2. Heat capacity jump at melting of collagen in 0.5M acetic acid

0.5M acetic acid and in water are: ΔC_p=0.2 Jg^{-1}K^{1} and ΔC_p=0.46 Jg^{-1}K^{-1} respectively.

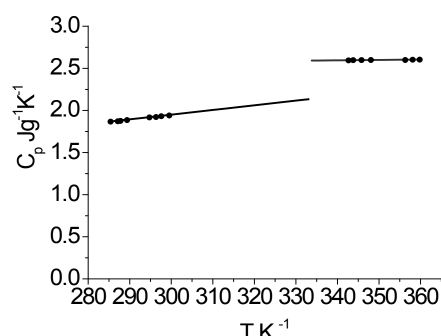

Figure 3. Heat capacity jump at melting of collagen in water

Figure 2 and Figure 3 present the temperature dependencies of collagen heat capacity in acetic acid and in water. Prior to transition, the heat capacity of collagen in acetic acid is higher than in water. The temperature coefficient of heat capacity in acetic acid is three times higher than that in water. Heat capacity jump at the denaturation in

After denaturation, the heat capacity of collagen in acetic acid and in water sligthly depend on temperature and is equal to ~ 2.62 Jg^{-1}K^{-1}.

In table are given the results of measurements some thermodynamic characteristics of rat tail tendon collagen obtained by calorimetric measurements in water and 0.5 M CH_3COOH.

Table 1.

Environment	**Denaturation temperature °C**	**Denaturation Enthalpy $\Delta H_r(T_d)$ Jg^{-1}**	**Heat Capacity Jump $\Delta C_p(T_d)$ $Jg^{-1}K^{-1}$**
Water	63 +/–0.2	60.1 +/–0.3	0.46 +/–0.02
0.5M Acetic acid	40.8 +/–0.2	50.2 +/–0.3	0.20 +/–0.02

Discussion

Acid solutable collagen exists in the form of a triple helix in the pH interval from pH = 2.0 to Ph = 4.0, which can be represented approximately in the form of elongated sticks. The researchers estimated the average molecular parameters of collagen macromolecules by different methods such as light scattering [23], statistical processing of electron-microscopic data [24], double refraction and viscosity [25]. The obtained average results were as follows: length 2800 A°, diameter 15 A°, molecular weight – 30000d. It should be noted that, in the interval of pH 2.0–4.0, the temperature of denaturation of collagen depends only slightly on the pH value. In water collagen exists as fibrils.

As it was mentioned above the problem of stabilization of collagen structure is one of the leading problems in physics and biology of collagen due to its functional importance connected with the phylogenetic change of thermal stability in the process of evolution [26; 27]. The main component of connective tissue – triple helix of collagen should be rather flexible on the one hand, in order to be able to form the fibrils spontaneously outside the cell under the physiological conditions, and, on the other hand, it should provide the formation of stable connective tissue capable to serve as mechanical functions. Both these properties are revealed in low temperature of denaturation of triple helix (T_d) in solutions within pH-2–4 interval, and in the

increase of the temperature of shrinkage of collagen fibers (T_s) [28].

The important role in formation of collagen fibers and growth of melting temperature *in vivo* together with other factors can play hydrophobic interactions the presence of which can be fixed and estimated by size of thermal capacity jump at the melting of collagen fibrils [2].

The difference between the temperatures of collagen fibers melting and denaturation of triple helix in the solution of different origin remains constant: 22 °C in average, as it was pointed above. Coiling into triple helix is achieved by intramolecular hydrogen bonds connecting the HN group of the glycine residue on one chain to a C=O group on an adjacent chain in the first position of collagen triplet $(Gly\text{-}X\text{-}Y)_n$ [29] by water molecules in the second position [30] and by the residues of hydroxyproline in the third position of triplet [31]. The further arrangement of collagen fibers in tissue takes place as a result of the change of surface charge of triplet helix, or by hydrophobic interactions between side-groups of amino acids [32; 33].

At present, the fundamental dependence of the enthalpy denaturation of collagen on the content of hydroxyproline and a slight change of enthalpy from 57.5Jg^{-1} to 58.2Jg^{-1} at the change of pH in 2–4 interval for the collagen of rat skin are determined[27]. This allowed one to predict the approximate value of heat capacity jump: 20 $Jmol^{-1}K^{-1}$ in the mentioned pH interval [2]. Hydrophobic interactions between the side groups of amino acids in the triple helix that can contact with each other, could be revealed by direct measuring of heat capacity jump at melting. According to our direct measurements the value of heat capacity jump for collagen of rat tile in 0.5 M acetic acid makes 0.2 $Jg^{-1}K^{-1}$, in agreement with above prediction, but the jump at denaturation of collagen fibers in the aqueous medium (0.46 $Jg^{-1}K^{-1}$) is twice and even more times bigger. A preliminary report about the existence of the specific heat jump of fibrils in water was made in [34]. The difference in the values of heat capacities jumps in water and in acetic acid may be explain by different nature of interactions of side chains of amino acid. It may be hydrophobic interactions nature, as in the literature there are data indicating the role of hydrophobic interactions at the **formation** of collagen fibers in mediums containing CH-groups with different degree of polymerization [30]. If we compare the results obtained by us with the results obtained for the heat capacity jumps in globular proteins, in which the hydrophobic interactions really determine the stability of globule, its value in collagen is of the same order, as in ribonuclease – the protein with the lowest level of hydrophobic interactions [35]. The relation between the value of heat capacity jump and the hydrophobic interactions is followed unambiguously on the example of globular proteins. For the majority of globular proteins, the hydrophobic interactions play the important role for the stability of globule in polar solvents. Such peculiarity of globular proteins is seen well in inversely proportional dependence of the enthalpy of collagen denaturation on the value of heat capacity jump [36; 37].

The facts, determined by us consisting in that both the enthalpy and heat capacity jump at melting of fiber in water are higher than that at melting of collagen in the solution of acetic acid make basis for the assumption that the hydrophobic interactions do not play such remarkable role in the increase of melting temperature, as it was in the case of globular proteins [35; 36]. Logically it seems more reasonable to assume that in the increase of the temperature of melting, the more important role can have the electrostatic interactions between the side-groups of amino acids, though the definite value of jump does not exclude the role of hydrophobic interactions. The obtained results give the definite idea about the physical-chemical nature of formation of collagen fibrils, but of course, the further investigations are needed to show the real process of fibril genesis.

Conclusions

1. By using the high precision pulsed differential scanning calorimeter (PDSC) the jumps of heat ca-

pacity at melting of collagen fibers of rat tails tendons in water and in 0.5M acetic acid were measured under the thermodinamically equilibrium conditions.

2. It is shown that the jumps of heat capacity of rat tail tendons collagen in water are twice as large as in 0.5M acetic acid.

3. The enthalpy of denaturation of rat tail tendons collagen in water is more than in acetic acid too.

4. The obtained results are discussed in the light of existing idea about the mechanism of fibril formation.

5. On the basis of the increase both enthalpy and the heat capacity jump at the melting of collagen of rat tail tendons in aqueous medium, as compared to the enthalpy and the heat capacity jump at denaturation in acetic acid, it is assumed that in the process of formation of these collagen fibrils the participation of electrostatic interactions of side groups of amino acids is probably more important than the participation of hydrophobic interactions.

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