Denaturation of Type I Collagen Fibrils Is an Endothermic Process Accompanied by a Noticeable Change in the Partial Heat Capacity†

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ABSTRACT: Thermal transitions of type I collagen fibrils were investigated by differential scanning calorimetry and spectrophotometry of turbidity within a wide range of external conditions. The advanced microcalorimeter allowed us to carry out the measurements at low concentrations of collagen (0.15−0.3 mg/mL). At these concentrations of collagen and under fibril-forming conditions, the melting curves display two pronounced heat adsorption peaks (at 40 and 55 °C). The low-temperature peak was assigned to the melting of monomeric collagen, while the high-temperature peak was assigned to the denaturation of collagen fibrils. It was shown that the denaturation of fibrils, in contrast to the monomeric collagen, is accompanied by a noticeable change in the partial specific heat capacity. Surprisingly, comparison of the collagen calorimetric curves in the fibril-forming and nonforming conditions revealed that ΔC_p of fibril denaturation is caused by a decrease in the C_p of collagen at premelting temperatures. This suggests the existence of an intermediate structural state of collagen in a transparent solution preceding fibril formation. Our study also shows that collagen fibrils formed prior to heating have thermodynamic parameters different from those of fibrils formed and denatured during heating in the calorimeter. Analysis of the data allowed us to determine the denaturation enthalpy of the mature fibrils and to conclude that the enthalpy plays a more important role in fibril stabilization than was previously assumed. The observed large ΔC_p value of fibril denaturation as well as the difference between thermodynamic parameters of the mature and newly formed fibrils is readily explained by the presence of water molecules in the fibril structure.

Long-time interest and numerous studies of collagen are mainly explained by its abundance and important function in the living organisms, involvement in a number of human hereditary diseases, and perspectives of the artificial collagen-like materials. The formation of a collagen fibril is the key event of all these processes. Most *in vitro* investigations of fibril formation have been focused on the fact that type I collagen molecules have the ability to self-assemble from solution (*1*). By now, the dependence of fibril formation on pH, ionic strength, and temperature is well established (*2*−*5*). Most of the studies suggested that collagen fibrils are stabilized mainly by changes in the entropy of the solvent (*6*−*11*). In accordance with these studies, the enthalpy of fibril formation is small or not observed at all (*7*, *10*, *12*). At the same time, it was shown that precise determination of the enthalpy may be hampered by the fact that the temperature of fibril formation coincides with the temperature of denaturation of monomeric collagen (*11*). As concerns fibril denaturation, it occurs at higher temperatures than the denaturation of monomeric collagen (*7*, *10*, *12*, *13*). Depending on the conditions and methods of collagen preparation, the resulting curves of fibril denaturation were different. For example, the number of transition peaks assigned to fibril denaturation ranged from one peak (*13*−*15*) to three peaks (*10*). These results were poorly reproduced at high concentrations of collagen due to its nonspecific aggregation. Thus, the process of fibril denaturation is still not clearly understood. This encouraged us to undertake another thermodynamic study of collagen fibrils using advanced scanning microcalorimeters which have a wide range of temperature regimes and allow measurements at low concentrations of collagen. Our interest was also based on the fact that self-association of collagen is a unique case for the calorimetric studies because both fibril formation and denaturation occur within a rather narrow interval of temperatures (*2*−*5*) and hence can be detected in one run of the calorimeter. Another reason for our interest was a possibility to gain insight into the structure of the collagen fibril. Until now, neither the pathway of fibril self-assembly nor the molecular packing of collagen within the fibril is completely understood (e.g., *16*−*18*). Here, we are reporting the results of our thermodynamic study and their possible structural interpretations.

MATERIALS AND METHODS

Acid-soluble collagen was extracted from white rat skin and purified using the procedure described earlier (*19*). To make sure that the sample contains only collagen type I and not a mixture of type I and type III collagen, we performed

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an amino acid composition analysis. Prior to the test, the sample was carboxymethylated by monoiodoacetic acid (*20*) and then hydrolyzed with 5.6 N HCl. Unlike type III collagen, type I collagen does not have cysteine residues. The analysis showed the absence of sulfhydryl groups and, therefore, of type III collagen in our sample.

Solutions of the monomeric form of collagen were prepared by dilution of the concentrated collagen solution in 0.007 M acetic acid, pH 3, and dialyzed against the same solvent overnight. The procedure and condition of fibril formation used in this work were described earlier (*3*, *21*). Fibril formation was initiated by 2-fold dilution of the collagen solution kept at pH 3.0 by a double-strength buffer obtained after mixing 0.225 M NaCl, 30 mM Na−P, and 30 mM Mes. After that, the pH was adjusted with concentrated NaOH to the needed value. All procedures were made at 5 °C. The concentration of collagen in the experiments was 0.15−0.3 mg/mL and was measured by a Perkin-Elmer spectropolarimeter using the known specific optical activity of collagen (*22*). The turbidity was measured by a HITACHI 124 spectrophotometer as the change in optical density at 313 nm. The cells were thermostated, and the cell temperature was measured by a Hewlett-Packard 2801A quartz thermometer. To prevent formation of Schiff base-mediated cross-links, sodium borohydride reduction of collagen was carried out as described earlier (*3*, *23*).

The calorimetric measurements were made on a DASM-4A precision scanning microcalorimeter (SCB, Pushchino, Russia) with a 0.5 mL gold capillary cell and on a new precision adiabatic scanning microcalorimeter SCAL-1 with 0.33 mL glass capillary cells (SCAL Co., Ltd., Pushchino, Russia). The calorimeters allowed us to measure accurately samples with a concentration of collagen from 0.15 to 0.3 mg/mL. During measurements, the rate of temperature was constant and equal to 1 K/min. The choice of the heating rate was governed by the collagen concentration in the sample and the sensitivity of the calorimeter. We chose a minimal rate when the calorimeter still could reliably register the melting curves of the sample with such a low concentration of collagen. At the same time, the increase of the collagen concentration caused poorly reproducible results. The specific partial volume of collagen was taken 0.700 cm^3 (*24*), and the molecular mass was 290 kDa.

RESULTS AND DISCUSSION

Dependence of Calorimetric Curve and Turbidity on pH. To check how pH affects the denaturation of collagen, first we recorded the calorimetric curve when collagen is known not to form fibrils (in 0.007 M acetic acid, pH 3.0). Under this condition, the melting curve displays one absorption peak at 40.3 °C with a specific enthalpy value of 72.3 ± 0.08 $J \cdot g^{-1}$ (Figure 1) that is in good agreement with our previous results (*19*). Next measurement was done at pH 6.0 using the buffer system 30 mM Mes, 30 mM Na−P, 0.225 M NaCl. The profile of the second calorimetric curve was identical to the first one (Figure 1). In parallel, spectrophotometric turbidity measurements were done under the same conditions. The collagen solution was transparent at pH 3.0 over the same range of temperatures (Figure 1). Interestingly, turbidity curves of collagen at pH 6.0 differed greatly. The turbidity rapidly increased, beginning at 32 °C and

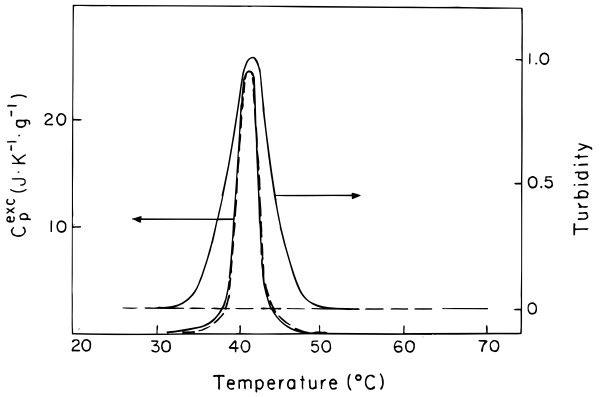

FIGURE 1: Temperature dependence of the excess heat capacity and turbidity (313 nm) of collagen at pH 3.0 (---) and pH 6.0 (—).

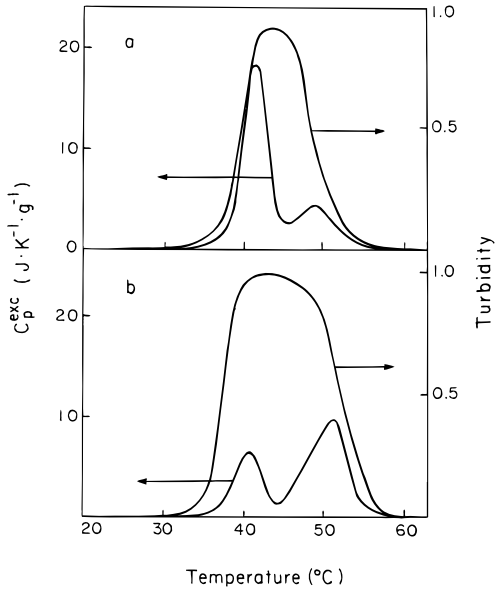

FIGURE 2: Temperature dependence of the excess heat capacity and turbidity of collagen at pH 6.75 (a) and pH 7.3 (b).

reaching the maximum at 40 °C. After denaturation of collagen, the turbidity decreased and, finally, disappeared at 50 °C (Figure 1). Electron microscopy showed that the solution turbidity corresponds to the formation of native collagen fibrils (*3*, *4*, *21*, *25*). So, the appearance of turbidity and, hence, fibril formation at pH 6.0 did not change the curve of thermal denaturation as compared with the denaturation of monomeric collagen at pH 3.0. It appears that these results support the widely accepted conclusion that the association of collagen into fibrils is an entropy-driven process. This may be true for poorly advancing self-assembly, as is the case at pH 6.0; however, our further studies at pH values from 6.5 to 7.5 make this conclusion arguable. The increase of pH leads to the appearance of the second peak at 50 °C (Figure 2). Furthermore, the increase of pH leads to a simultaneous decrease of the first peak and an increase of the second one. As concerns the turbidity, it also appears practically simultaniously with the first calorimetric peak, and then the collagen solution becomes transparent at higher temperatures just after the second calorimetric peak.

Assignment of Two Heat Absorption Peaks on Calorimetric Curves. The important question to be solved was to assign each of the two heat absorption peaks of the calorimetric

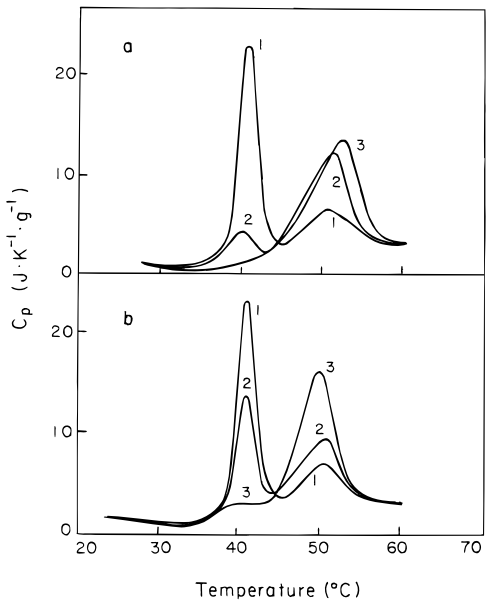

FIGURE 3: (a) Temperature dependence of the partial heat capacity of collagen at pH 7.45 and $c = 0.32$ mg/mL at different conditions of measuring. Curve 1: The scanning began at the moment when the collagen solution was placed in the calorimetric cell. Curve 2: Prior to measurement, the collagen solution was kept for 4 h at 5 °C. Curve 3: The scanning was performed after keeping the collagen solution for 50 min at 26 °C in the calorimeter cell with subsequent cooling to 3 °C. (b) Partial heat capacity of collagen solutions at pH 7.3 and different concentrations: (curve 1) $c = 0.17$ mg/mL, (curve 2) $c = 0.22$ mg/mL, (curve 3) $c = 0.44$ mg/mL.

curve to the formation/destruction of this or that structural form of collagen. Previous data (*19*) indicated that the low-temperature transition is related to the melting of the monomeric collagen. Our experimental data suggest that the high-temperature peak corresponds to the melting of the fibrillar form of collagen. Indeed, the turbidity of the collagen solution, that reflects the degree of fibril formation, disappears during the second transition. It is also known that collagen fibrillogenesis is a time-dependent process and its lag period is comparable with the duration of the calorimetric measurements (*3*, *5*, *21*). So, it was reasonable to check the dependence of the calorimetric curve on the time of preincubation under fibril-forming conditions. Our measurements showed that the first peak decreased as the second peak increased with the time of preincubation (Figure 3a). This supports the assignment of the second transition to fibril denaturation. Of special interest is the case when the collagen was kept 50 min at 26 °C in the calorimeter cell in order to get mature fibrils prior to heating. Then, after cooling to 3 °C, this sample was melted. In this case, the low-temperature peak disappeared, and only one high-temperature peak was observed (Figure 3a, curve 3). This melting curve did not change after keeping the collagen solution at 26 °C for more than 50 min; this allows us to assume that the collagen fibrils are "fully maturated" after 50 min. It is also known that the rate of fibril formation is directly proportional to the collagen concentration (*4*, *21*, *25*). Our study shows that the higher the concentration of collagen, the lower the first peak and the greater the second peak (Figure 3b). This fact confirmed that the second transition is related to fibril destruction. Thus, it is possible to conclude that the first peak corresponds to the denaturation of monomeric collagen and the second one to that of collagen fibrils. An important point is that the two-peak melting curve means denaturation of collagen molecules in monomeric and fibrillar states rather than denaturation of two different domains of a collagen molecule.

Table 1: Thermodynamic Characteristics of Thermal Denaturation of Collagen[a]

pH	T_{d1} (K)	T_{d2} (K)	Q_{d1} (J/g)	Q_{d2} (J/g)	Q_{tot} (J/g)	ΔT_1 (K)	ΔT_2 (K)	ΔC_p [J/(K·g)]
3.0	313.9	–	72.3	–	72.3	2.4	–	–
6.0	313.9	–	71.5	–	71.5	2.4	–	–
6.3	314.1	–	72.3	–	72.3	2.4	–	–
6.5	314.0	–	73.6	–	73.6	2.4	–	–
6.75	313.9	321.7	63.5	10.9	74.4	2.4	4.5	0.38
7.0	313.9	322.5	58.9	18.0	76.9	2.4	4.5	0.63
7.15	313.1	322.8	53.1	28.8	81.9	2.4	4.5	1.05
7.3	313.2	323.2	48.5	37.2	85.7	2.4	4.5	1.25
7.55	313.7	323.9	30.5	60.6	96.1	2.4	4.5	1.46
7.44[b]	–	326.2	–	97.4	97.4	–	3.3	1.60

[a] T_{d1} is the temperature of the first transition. T_{d2} is the temperature of the second transition. Q_{d1} is the heat absorption of the first transition. Q_{d2} is the heat absorption of the second transition. Q_{tot} is the total denaturation of heat absorption under transition. [b] The collagen solution was kept for 50 min at 26 °C at pH 7.44.

Relationship between the Half-Width of the Thermal Transition and the Cooperative Regions of Collagen. For interpretation of calorimetric data, it is important that the collagen denaturation can be considered as being in principle reversible (*26*, *27*). It has been shown (*28*, *29*) that strand separation on collagen melting takes place only at the final stage of the denaturation. Numerous independent studies (e.g., *30*−*32*) suggest that just this last stage is responsible for the observed irreversibility of the collagen transition. Therefore, the main process of collagen disruption can be regarded in principle as an equilibrium reaction.

The melting curves display two pronounced heat absorption peaks which enabled a rather accurate direct determination of the thermodynamic parameters characterizing each transition. The values of these parameters obtained at the same concentrations of collagen (0.15−0.3 mg/mL), rate of calorimetic measurements (1 K/min), buffer conditions, and variable pH are shown in Table 1. The analysis of our data shows that the melting temperature (T_d) and half-width (ΔT) of both thermal transitions are constant within the studied pH range (Table 1). Assuming that a molecule is represented as a set of cooperative blocks which melt as single units (see, e.g., *26*), this result implies that the size of the cooperative blocks is the same at different pHs. The effective number of cooperative blocks (n) corresponding to the thermal transition can be calculated by the formula:

$$n = QM\Delta T/4RT_d^2 \qquad \text{(I)}$$

where M is the molecular weight of collagen, R is the gas constant, and Q is the heat transition normalized to the amount of denaturated collagen per gram. As shown in Table 1, the heat of the first transition (Q_1) decreases and the heat of the second one (Q_2) increases with pH. These heat values being used in formula I will give a variable value of n. At first glance, this is in contradiction with the previous conclusion about the invariability of n corresponding to each of two transitions. However, taking into account that

collagen molecules are distributed between the monomeric and fibrillar states at the fibril-forming pH, this contradiction may be easily resolved. In accordance with our assignment, the first peak corresponds to thermal denaturation of monomers, and the second peak corresponds to denaturation of collagen within the fibril. Therefore, the portion of collagen in the monomeric and fibril states can be approximately assessed by the relation between areas under the two heat absorption peaks. If so, the values of Q_1 and Q_2 normalized to the amount of monomeric and fibrillar collagen, correspondingly, become constant within the considered pH range. As a result, the calculated number of cooperative blocks corresponding to the denaturation of monomeric collagen is constant, being 19 both in the fibril-forming conditions and at the pH when fibrils are not formed. One such cooperative unit involves approximately 210 amino acid residues, i.e., 70 residues per chain. This is in agreement with the previously determined values for the collagen triple helix (*19*). The half-width of the high-temperature peak is also constant, being 4.5 °C. The number of cooperative units, calculated by formula I using the amount of the fibrillar collagen assessed from the peak areas, increases from 40 to 50 at pH from 6.75 to 7.55. This gives an unexpected result that the size of the cooperative blocks of collagen in the fibril is about twice smaller (30 residues per chain) as compared with that of the monomer. However, obviously the cooperative block theory cannot be directly applied to such a complex object as a collagen fibril. The width of the fibril transition may be determined by other reasons, for example, by variety of the molecular packing within the fibril. Also, the observed asymmetry of the second heat absorption peak (Figures 2 and 3) suggests that the melting of the fibril is a multistep process. It is significant that the half-width 4.5 °C of the high-temperature transition peak of the collagen heated without preliminary fibril assemby decreases to 3.3 °C for the "fully mature" fibrils (Figure 3a, curve 3, and Table 1).

Change in the Partial Heat Capacity of the Collagen Fibril. It is known that the collagen monomer melts almost without any change in the partial heat capacity (ΔC_p) (*19*, *33*). However, upon melting of collagen fibrils, a remarkable ΔC_p value is observed (Table 1). The increase of ΔC_p with pH correlates with growing of the heat absorption peak corresponding to the denaturation of the collagen fibril. Knowing the portion of fibrillar collagen (k) in the total amount of collagen (estimated as $1 - Q_{d1}/Q_m$ where Q_{d1} is the absorption heat of the first transition estimated directly from the area under the peak, Q_m is the known absorption heat of the monomeric collagen), it is possible to calculate ΔC_p of the collagen fibril denaturation by the formula:

$$\Delta C_p = N^{-1}\sum \Delta C_{pi}/k_i \qquad \text{(II)}$$

where N is the number of measurements, i is the measurement at a certain pH, and k is the portion of collagen in the fibrillar state. In accordance with this calculation, ΔC_p is 3.4 ± 0.8 J K^{-1} g^{-1}.

The reason for the noticeable ΔC_p value of collagen melted at the fibril-forming pH as compared to the ΔC_p value of the collagen denatured at non-fibril-forming pH was not clear. The paradox of these observations lays in the fact that at both pHs, collagen is likely to have the same initial

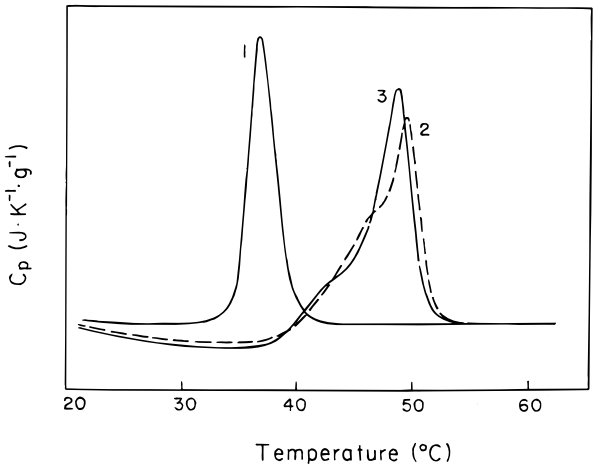

FIGURE 4: Temperature dependence of the partial heat capacity of collagen treated by sodium borohydride and transferred to different conditions, $c = 0.18$ mg/mL. Curve 1: pH 2.9, 0.5 M acidic acid. Curve 2: pH 7.3, the scanning began at the moment when the collagen solution was placed in the calorimetric cell. Curve 3: Recorded after the following procedure: the scanning began after keeping the collagen solution for 50 min at 26 °C in the calorimetric cell with subsequent cooling to 3 °C.

states (at 4 °C). Therefore, we first hypothesized that in the fibril-forming conditions, collagen has a denatured state with a higher C_p value than that of collagen monomer. There was one plausible reason, explaining why the final states may be different. It is known that collagen molecules can form Schiff base mediated cross-links even during the short time of being in aggregates (*3*, *23*). Therefore, in principle, collagen, being for some time in the fibrillar state, can have a covalently cross-linked denatured state with a different C_p as compared with the denatured state of the monomer. To test this hypothesis, we melted collagen under fibrillogenesis-permissive pH and conditions when the cross-links are not formed. The reduction conditions did not change the calorimetric curve (Figure 4), and this denied the hypothesis. This also points out that the difference of the premelting states of the collagen under the fibril-forming and nonforming pH is more probable. As shown in Figure 4, where the asymptotes of the denatured states are superimposed, at the fibril-forming pH, the C_p value of collagen decreases with temperature in the premelting region compared to the monomeric collagen. Although both collagen solutions were transparent at this temperature, the difference in C_p suggests that at the fibril-forming pH, collagen molecules are probably already self-associated. The conclusion about the intermediate collagen associates is in agreement with earlier publications which conclude, using other methods, that the formation of the fibril proceeds through initiation in which intermediate aggregates are formed (*3*, *11*, *21*, *25*).

In proteins, the magnitude of ΔC_p is usually proportional to the change in the number of contacts between hydrophobic groups (*34*). The decrease of C_p during formation of the intermediate associates should be determined by formation of intermolecular contacts between collagen side chains in the aggregate, compared to the monomeric state where collagen side chains are exposed to water. However, there are two observations which cannot be explained using the theory of hydrophobic contacts. First, the magnitude of the observed ΔC_p value cannot be explained only by the hydrophobic contacts. This contradiction may be resolved

if water molecules located within the fibril are taken into consideration. The detailed analysis of the intermolecular interactions (*17*, *35*, *36*) showed that in contrast to aggregates of α-helices and *β*-sheets, where complete displacement of water occurs, in the aggregates of collagen molecules water is displaced from the intermolecular space partially. Structuring of water molecules causes a decrease in the C_p value in addition to the hydrophobic contacts. This structural interpretation also fits well the thermodynamic data on the collagen-like part of C1q (*37*). The second observation which cannot be explained by hydrophobic contacts concerns the "fully mature" collagen fibrils. The ΔC_p of this fibril (1.60 J K^{-1} g^{-1}) is less than the ΔC_p which was assigned to the prefibrillar aggregates formed in the transparent collagen solution (3.4 J K^{-1} g^{-1}) (Table 1). This fact is difficult to understand either from the theory of hydrophobic contacts or from the explanation based on the triple-helix flexibility, which state that the mature collagen fibril should be less compact and more flexible than its intermediate associates. At the same time, the explanation using structural water can easily resolve this problem. Indeed, the intermediate collagen associate is assumed to have less dense protein packing, i.e., a larger amount of structured water molecules in the interhelix space. When the fibril is formed, the collagen packing becomes more tight, and, as a consequence, the amount of ordered water between the collagen molecules decreases. This may result in increasing the C_p of the mature fibril compared to the fibrillar intermediates.

Our structural interpretation is in line with the temperature dependence of the collagen conformation obtained by Fourier transform infrared spectroscopy under fibrillogenesis-permissive conditions (*38*). This study showed that the triple helix is perfected during the lag phase and then again distorted after fibril formation. The perfection of the triple helices may be explained by formation of the water-rich intermediate collagen associates, while the subsequent distortion of the helical conformation is probably due to the adaptation of molecules to the more tight packing within the fibrils.

Enthalpy of Fibril Denaturation. The increase of pH causes an increase of the total absorption heat (Table 1). Analysis of the data presented in Table 1 shows that the increase of the total denaturation heat can be approximated by the equation:

$$(Q_{tot})_i = Q_m + (T_{d2}{}^i - T_{d1}{}^i)\Delta C_{pi} \qquad \text{(III)}$$

where *i* corresponds to the measurement at a certain pH value and Q_m is the transition heat of monomeric collagen. In most of our measurements, collagen molecules formed fibrils and dissociated again during one run of the calorimeter. In this condition, it is practically impossible to determine the heat of fibril formation, if any, because the formation and destruction are supposed to have nearly equal small values and opposite signs that should hide the resulting effect. However, an interesting result has been obtained when the "fully mature" collagen was melted. The principal difference in this experiment is that when the melting begins from the fibrillar state, it is possible to separate the thermodynamic parameters of the fibril denaturation from the melting of the triple helix itself. This measurement shows that the mature collagen fibrils melt at 326.2 K as one asymmetric peak with the absorption heat of 97.4 J g^{-1}. Since the melting enthalpy of collagen molecules does not depend on pH and is equal to 72.3 J g^{-1} at 313.9 K (Table 1), it is possible to estimate the denaturation enthalpy of fibril denaturation by the formula:

$$Q_f = Q_{tot} - Q_m - \Delta C_p{}^m(T_{d2} - T_{d1}) \qquad \text{(IV)}$$

in accordance with which $Q_f = 97.4 - 72.3 - 0.08$ (326.2 − 313.9) = 23.8 J g^{-1}. This points out that despite numerous studies which showed that collagen fibril formation is an entropy-driven process accompanied by negligibly small changes in the enthalpy value, our data indicate that the collagen fibril may be stabilized significantly at the latest phase of the fibrillogenesis due to the change in enthalpy.

In conclusion, it was shown that the denaturation of the fibrils is accompanied by a noticeable change in the ΔC_p in contrast to monomeric collagen. This result, taken together with the data on the collagen-like region of C1q (*37*), allows us to make a general conclusion that the self-association of the collagen causes a decrease in the partial heat capacity. This phenomenon can be explained by the following structural changes: formation of the side chain contacts between the collagen molecules and ordering of water in the intrafibrillar space. The other conclusion is that the final stabilization of fibrils, i.e., maturation, is an enthalpic process as compared to the earlier entropy-driven stages of collagen self-assembly. The maturation can be explained by more compact packing of the collagen molecules caused by the release of water from the intrafibrillar space, which should result in a decrease of the enthalpy of the collagen fibrils. We showed that during formation of the fibrils the decrease of C_p occurs at the early phase of the process, when the collagen solution is still transparent. In terms of molecular structure, this effect can imply the existence of intermediate aggregates where collagen molecules form numerous side-by-side contacts. Among most probable intermediate structures which meet these conditions, we would mention the previously described bunches of collagen molecules (*17*, *39*−*41*) or collagen microfibrils (*16*, *42*, *43*).

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