Thermal (in)stability of type I collagen fibrils.

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We measured Young’s modulus at temperatures ranging from 20 to 100°C for a collagen fibril taken from rat’s tendon. The hydration change under heating and the damping decrement were measured as well. At physiological temperatures 25 – 45°C Young’s modulus decreases, which can be interpreted as instability of collagen. For temperatures between 45 – 80°C Young’s modulus first stabilizes and then increases with decreasing the temperature. The hydrated water content and the damping decrement have strong maxima in the interval 70 – 80°C indicating on complex inter-molecular structural changes in the fibril. All these effects disappear after heat-denaturating the sample at 120°C. Our main result is a five-stage mechanism by which the instability of a single collagen at physiological temperatures is compensated by the interaction between collagen molecules within the fibril.

PACS numbers: 36.20.-r, 36.20.Ey

Type I collagen is the major structural element in the extra-cellular matrix. The native state of collagen is made up by three polypeptide chains, which are twisted together into a triplex [1]. Naively, the collagen triplex is expected to be very stable, since it forms fibrous connective tissues of bones, skin and tendons; see Fig. 1 for the tendon hierarchical structure. But in contrast, the collagen triplex denaturates into separate chains at the helix-coil transition $T_{hc}$, which for mammals and birds is close to [2] — or even lower from [3] — the body temperature (for poikilothersms $T_{hc}$ relates to the upper environmental temperature). Much attention was devoted to the physiological meaning of this marginal thermal (in)stability [3] [4] [5], which is generally explained as compromising between the instability of a single collagen versus flexibility of collagen fibrils [4] [5]. However, it is so far not clear by which specific mechanisms marginally unstable collagen molecules achieve to form the stable collagen fibril.

We approach to this problem by studying thermal denaturation of a collagen fibril via its mechanical characteristics such as Young’s modulus, logarithmic decrement of damping, and the hydrated water content. The Young’s modulus of collagen triplexes and fibrils was measured via various methods [6] [7] [8]. The hydrated water was found to be essential for maintaining the triplex [9] [10]. It is also believed to be important for the structure of fibril, though direct experimental evidences for this are lacking [10]. Combining these quantities enables us to contrast features of a single collagen molecule to those of a fibril, and study the impact of the intermolecular interaction on the fibril stability. The structural transformations in the fibril are determined by competition between entropy increase versus the intermolecular interactions [7]. It is known that for such materials the mechanical properties can provide information not only on internal elastic forces, but also on the molecular processes involving stress relaxation [11]. In particular, these properties can uncover relaxation processes both in the main polymer chain and side groups, e.g., they revealed the phenomenon of low temperature glass transition in the surface layers of protein molecules [12].

We shall study the viscoelastic properties of collagen fibrils for a wide temperature range 20 to 100°C and at frequencies within the eigen-frequency domain (50 – 20000 Hz). The collagen fibril is composed of collagen triplexes and hydration water with a small amount of salts. Fig. 1 shows a simplified scheme of the collagen hierarchy from the separate triplex to the tendon. Our mechanical methods allow studying samples with diameter 1 µm, and we shall work with a separate fibril (denoted by F in Fig. 1). See Refs. [13] for optical methods.
of studying the collagen structure.

Materials and Methods. Achilles tendons of young rats were obtained from the Yerevan Medical Institute. Separation of fibrils from the fiber and from each other was carried out mechanically in 96% of ethyl alcohol at temperature 5°C using micro-tweezers and microscope. The experimental sample is a cylinder of length 0.3 mm, which is cut-off from a separate fibril, held in the micro-tweezers and washed out in distilled water before experiments. More details on the preparation of similar experimental samples are found in [12].

The sample under investigation was enclosed in the experimental chamber and placed in a temperature-controlled cabinet with the temperature maintained at 25°C. The hydration level of the sample was adjusted by placing a drop of CaCl$_2$ solution at the bottom of the experimental chamber. The sample was allowed to equilibrate at a given humidity for several hours. The relative humidities from 97 to 32% in the chamber were achieved by means of CaCl$_2$ solutions of different concentrations, while the relative humidities 15% and 10% were obtained via saturated solutions of ZnCl$_2$ and LiCl, respectively.

The chamber was then covered by the heat-insulating jacket and placed on the table of the microscope used to measure the sample vibration. The viscoelastic properties of the sample length were measured point by point when varying the temperature continuously at a rate of 1°C/min. For checking the features of hysteresis and irreversibility (see below) the heating rate was occasionally reduced to 0.1°C/min.

For measuring the Young’s modulus $E$ (defined as the ratio of stress [pressure] over strain) and the logarithmic damping decrement $\vartheta$ we applied Morozov’s micromethod [16]. The method is based on the analysis of electrically excited transverse resonance vibrations of the sample (fibril cylinder), which is cantilevered from one edge (another edge is free). Modifications of the method that enable measuring $E$ and $\vartheta$ within a wide temperature range is described in [12, 17].

As a characteristic of internal friction we employ the logarithmic decrement of damping

$$\vartheta = \ln[A(t)/A(t+T)],$$

where the oscillation amplitude $A(t)$ of the sample has two consecutive peaks at times $t$ and $t+T$. For measuring $E$ and the phase-frequency or the amplitude-frequency characteristics of oscillations (employed for obtaining $\vartheta$), it is necessary to change smoothly the frequency $f$ of the induced oscillations and determine the basic resonance frequency, which corresponds to the maximal oscillation amplitude of the sample free end. Young’s modulus for sample main axis is calculated by following formula [15]

$$E = 3.19 \cdot f_0^2 \cdot l^4 \cdot \rho \cdot P/I_{\text{min}},$$

where $f_0$ is resonance frequency, $l$ is the sample length, $P$ is the cross-section area, $\rho$ is the density, and $I_{\text{min}}$ is the main inertia moment of that section, which corresponds to the deformation plane with the minimal stiffness. For the round cross-section of our samples $I_{\text{min}} = \pi \cdot D^4/64$ [15] and $P = \pi \cdot D^2/4$, where $D$ is the sample diameter (measured with precision 0.02 $\mu$m). Thus Young’s modulus is calculated from (2), where $l$, $\rho$, $P$ and $I_{\text{min}}$ are the known sample characteristics and $f_0$ is measured on the experiment.

The data on the hydration water content is obtained by measuring the sample mass via the method of [20]. This method allows to detect the changes of mass within 0.00001 mg in microsamples weighting up to 0.01 mg.

Results and Discussions. The main contribution to Young’s modulus of collagen fibril comes from the rigid-
ity of separate collagen molecules and from the inter-

molecular interactions. It was argued recently that the
interplay between these two mechanisms is the key for
understanding the collagen mechanics [18].

Fig. 2 displays the Young modulus of the collagen fibril
versus temperature. At the initial temperature 25°C we
created relative humidity 93%. The water content in the
fibril is 0.3 g H₂O/g dry collagen.

For the considered frequency range (50−20000 Hz) the
studied quantities (e.g., Youngs modulus) does not show
any significant dependence on the frequency (not shown
on figures). Thus we are in the slow deformation regime,
e.g., the values of Youngs modulus shown in Fig. 1 are
consistent with earlier results in this regime [6] [7] [8]. It is
however expected that dependence on the frequency will
show up for larger frequencies (e.g., the Youngs modulus
starts to increase with frequencies) [6]. For our samples
this dependence starts above 100 KHz. In the studied
frequency range the hydrated water does not contribute
directly to the Youngs modulus [19] [20], but the water
can induce structural changes in the fibril that will alter
its elastic features.

As suggested by our results, the studied temperature
domain 20 − 100°C should be separated into five inter-
vals; see Fig. 2. For each of these intervals we discuss the
behavior of the measured quantities for the native colla-
gen fibril sample and compare it with the corresponding
heat-denaturated sample, which was prepared by keeping
the native sample at 100°C for 30 minutes.

1. Young’s modulus of the native collagen smoothly
decreases between 20°C and 45°C; see Fig. 2 (tempera-
ture borders of the intervals are defined conventionally).
There is no difference between the Young modulus of
the native sample and that of the heat-denaturated sam-
ple. In this temperature range the logarithmic decrement
of damping (LDD)—which characterizes internal friction
and is generally rather susceptible to inter-molecular in-
teractions—does not experience any systematic change;
see Fig. 3. The hydrated water content also does not
change in this interval; see Fig. 4.

Note that the conformational changes in this interval are
completely reversible, since the features of the fib-
ril did not change after repeating the cooling-reheating
process ten times. Though the Young modulus and the
LDD of the native fibril are almost indistinguishable from
those of the heat-denaturated fibril, the hydrated water
content (HWC) does show certain differences between the
native and heat-denaturated sample; see Fig. 3.

It is likely that in this stage only the triplex confor-
mation changes. We think that the decrease of Youngs
modulus in this temperature interval is a mechanic ana-
logue of very slow single triplex thermal instability, which
was calorimetrically observed in [3]. In one way or an-
other, similar instabilities are seen by many experiments
that work at very low concentration, so that the inter-
molecular interactions do not play any role [2].

2. In the temperature interval 45 − 58°C the de-
crease of Young’s modulus for the native sample is im-
peded as compared to the previous stage. Now differences
between the native and heat-denaturated situations set
in: the Young modulus of the native sample is larger
and decreases slower as compared to that of the heat-
denaturated sample. Also in this interval we noted first
indications of hysteresis and irreversibility during heat-
ing and recouling (not shown on figures). We checked
and confirmed these indications by changing the heating
rate from 1°C/min to 0.1°C/min.

Presumably already in this temperature interval the
intermolecular interactions influence on the change of
Young’s modulus. Recall that the collagen triplexes in
the fibril are tied with cross-links, direct hydrogen bonds
and water-mediated hydrogen bonds; see Fig. 1 (d).

3. The third interval lies in 58−75°C. Here the Young
modulus of the native sample is nearly constant. To our
knowledge such an effect was never seen for a single colla-
gen triplex. The Young modulus of the heat-denaturated
sample continues to decreases following the same linear
law as for the previous stages; see Fig. 2. The logarithmic
decrement of damping (LDD) and the hydrated water
content (HWC) of the native sample increase suddenly.
The endpoint of the interval (approximately 75°C) plays
a special role, since here Youngs modulus starts to in-
crease, while both LDD and HWC assume their maximal
values; see Figs. 3 and 4. In this interval the hysteresis
is more pronounced than for the previous interval.

Altogether, this seems to indicate that the inter-
molecular interactions start to play an important role.
Most likely reason for the sudden changes of LDD and
HWC—which we stress are clearly absent for the heat-
denaturated sample—is that partially molten collagen
molecules start to overlap and create new bonds between
each other. This facilitates intermolecular interactions.
The hydrated water content increases, since new adsorp-
tion centers open up during the melting. A similar corre-
lations between the presence of water and the strength of
inter-molecular interactions was obtained in Ref. [6] via
atomistic modeling.

Note that theoretical arguments predicted recently
that the energy dissipation in the collagen fibril gets max-
imized at the transition from homogeneous intermolecular
shear to slip pulses [18]. In our Figs. 2, 3 and 4 we
also see simultaneous indications of structural changes
(Young’s modulus and hydrated water content) and dis-
sipation maximization (LDD pick).

4. Between 75 − 80°C Youngs modulus of the native
sample starts to increase in sharp contrast to Youngs
modulus of the heat-denaturated sample that keeps de-
creasing; see Fig. 1. Simultaneously, both LDD and
HWC start to decrease; see Figs. 3 and 4. At the end of
this interval (i.e., at 80°C) Youngs modulus almost
approaches its initial value at 25°C.

We think that a possible reason for increasing Young’s
modulus is that the network of the inter-molecular bonds (established already during the previous stage) develops and contributed significantly to the rigidity. This can also expel the water out of the fibril.

We see that the crucial difference between the measured characteristics of the native and heat-denatured sample indicate on the existence of an important structural feature of the native fibril, which ensures its stability for temperatures larger than 58°C and which is absent for the heat-denatured sample.

Above 80°C the Young modulus keeps on increasing, though slower than for the previous step. The LDD stops decreasing and starts to change irregularly, in contrast to the LDD of the heat-denatured sample. The HWC in this region keeps on decreasing before 92°C, and then changes non-monotonously. The dynamics in this region is irreversible: if the heating stops at some temperature larger than 80°C and the sample is cooled back to 20°C, the above behavior of the native fibril is not recovered upon subsequent heating. Instead we obtain the heat-denatured behavior displayed on Figs. 2–4. On the other hand, if the heating simply stops at some temperature larger than 80°C, the sample slowly relaxes to the heat-denatured value of the Young modulus.

It is likely that the origin of this irreversibility is mainly entropic: there are already so many well-established inter-molecular bonds in this regime that the reverse transition to the weakly coupled inter-molecular situation becomes impossible. The HWC and LDD in this interval behave non-monotonously indicating on further structural changes, which are again absent for the heat-denatured sample.

In conclusion, we studied thermal stability of the (type I) collagen fibril via measuring its Young’s modulus, logarithmic decrement of damping (LDD) and hydrated water content (HWC). All the measurements were done in parallel for the native collagen fibril from rat’s tendon and its heat-denatured version. We aimed to understand how the instability of a single collagen triplex is assimilated by this structure. We observed that between 20°C and 50°C the Young modulus of the native fibril decays with increasing the temperature. This indicates on the (partial) instability of the fibril due to single-collagen effects. For higher temperatures the LDD and HWC indicate on serious structural changes in the fibril. Due to these changes Young’s modulus first becomes constant and then (upon further heating) increases with temperature. None of these effects is seen on the heat-denatured fibril, which displays monotonously decaying Young’s modulus and a relatively trivial behavior for LDD and HWC.

This work was supported by Auburn University Detection and Food Safety Center, NSF (Grant CTS-0330189 to ALS) and Volkswagenstiftung (to AEA).

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