Type I collagen is thermally unstable at body temperature

E. Leikina, M. V. Mertts, N. Kuznetsova, and S. Leikin*

National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

Edited by Donald L. D. Caspar, Florida State University, Tallahassee, FL, and approved December 3, 2001 (received for review June 17, 2001)

Measured by ultra-slow scanning calorimetry and isochemical circular dichroism, human lung collagen monomers denature at 37°C within a couple of days. Their unfolding rate decreases exponentially at lower temperatures, but complete unfolding is observed even below 36°C. Refolding of full-length, native collagen triple helices data 2°C below 30°C. Thus, contrary to the widely held belief, the energetically preferred conformation of the main protein of bone and skin in physiological solution is a random coil rather than a triple helix. These observations suggest that once secreted from cells collagen helices would begin to unfold. We argue that initial micromoulnding of their least stable domains would trigger self-assembly of fibers where the helices are protected from complete unfolding. Our data support an earlier hypothesis that in fibers collagen helices may melt and refold locally when needed, giving fibers their strength and elasticity. Apparently, Nature adjusts collagen hydroxyproline content to ensure that the melting temperature of triple helical monomers is several degrees below rather than above body temperature.

Type I collagen is the most abundant animal protein, and forms the matrix of bone, skin, and other tissues. One would think that matrix proteins should be very stable. Nevertheless, for over half a century it was taken for granted that triple helices of type I collagen melt just several degrees above body temperature (1, 2). Naturally, much attention was paid to the origins (3, 4) and physiological role (5) of this marginal thermal stability. It was proposed, for example, that destabilization of type I collagen by mutations is an important factor in osteogenesis imperfecta, a debilitating and often lethal hereditary disorder characterized by brittle bones (6, 7).

Most measurements of collagen denaturation were (and still are) done by scanning at ~0.02–2°C/min heating rate or after a short equilibration at constant temperature (usually several minutes). To infer the equilibrium melting temperature, T_m, one must extrapolate such data to zero heating rate or infinite waiting time (1). But a recent differential scanning calorimetry (DSC) study (8) and our DSC measurements at much slower rates (Fig. 1) unequivocally show that the apparent T_m changes linearly with the logarithm of the heating rate at all rates and equilibration times reported before. Because a logarithmic dependence cannot be extrapolated to zero argument (Fig. 1), the equilibrium T_m cannot be inferred from the published data. Either collagen denaturation is an intrinsically nonequilibrium process (8) or the equilibrium T_m is lower and the protein is less stable than previously believed.

In the present study we demonstrate that the equilibrium T_m of collagen does exist, but it is several degrees below body temperature in physiological solution. The thermodynamically preferred conformation of collagen at body temperature is a random coil rather than helix. This must be a deliberate design, because Nature tunes collagen stability to different body temperature of different species by adjusting hydroxyproline content of the helix (1). Apparently, it does this to keep collagen helix intrinsically unstable rather than stable. At the same time, the chaperone-assisted folding of the helix must still be possible and the helix should not unfold too fast to prevent fibrillogenesis. After presenting the data that have led us to these conclusions, we discuss implications of our findings for understanding collagen metabolism and collagen-related diseases.

Materials and Methods

Collagen Purification, Characterization, and Buffer Selection. Tails from young rats were purchased from Pel-Freeze Biologicals. Pepsin-soluble collagen was purified from tail tendons as described (9, 10). Human lung collagen was purchased from the Elastin Products Company and used without further purification. To characterize the protein and its proteolytic degradation during denaturation and renaturation experiments, small aliquots were analyzed by SDS/PAGE on precast gradient 3–8% Tris-Acetate NuPAGE gels (Invitrogen). For quantitative analysis, the gels were stained by SYPRO Orange (Molecular Probes) and scanned on a Fuji FLA3000 (Fuji Medical Systems) fluorescence scanner. From SDS/PAGE, the collagen extracted from rat tail tendons was virtually pure type I, whereas human lung collagen contained up to ~20–30% of type III molecules.

To inhibit fibrillogenesis during melting and refolding experiments, we used 0.2 M sodium phosphate/0.5 M glycerol buffer (pH 7.4) rather than traditional low pH buffers, because low-pH data are difficult to extrapolate to physiological conditions. Systematic measurements in mixtures of sodium phosphate with glycerol and sodium chloride at pH 7.4 revealed that the change in apparent T_m on variation of buffer composition is given by

$$\delta T_m = c_{\text{phosph}}[\text{Na}_2\text{HPO}_4] + c_{\text{gly}}[\text{glycerol}] + c_{\text{NaCl}}[\text{NaCl}],$$

where concentrations of buffer components are denoted by square brackets. The coefficients $c_{\text{phosph}} = 3.6 \pm 0.3°C/M, c_{\text{gly}} = 0.79 \pm 0.02°C/M, and c_{\text{NaCl}} = -3.8 \pm 0.1°C/M were the same for rat-tail tendon and human collagens. They were independent of the buffer composition and heating rate. To recalculate physiological T_m one can therefore subtract 1.7°C from the value measured in 0.2 M sodium phosphate/0.5 M glycerol.

To extend the equilibration times up to several weeks at or above room temperature, a protease inhibitor mixture (Roche Molecular Biochemicals, #1697498) was added to protein solutions used for circular dichroism (CD) measurements to prevent slow proteolytic degradation. The mixture had no effect on the denaturation or renaturation kinetics. The extent of collagen degradation was monitored by SDS/PAGE analysis of small sample aliquots as described above.

Differential Scanning Calorimetry (DSC). DSC thermograms of 0.1–3 mg/ml collagen solutions in different buffers were recorded in a Nano II differential scanning calorimeter (CSC, Chicago) at

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: DSC, differential scanning calorimetry.

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*To whom correspondence should be addressed: National Institutes of Health, Building 12A, Room 2041, 12 South Drive MSC 5626, Bethesda, MD 20892-5626. E-mail: leikin@helix.nih.gov.

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0.004–2°C/min heating rates. The apparent melting temperature was defined at the maximum of the melting peak after baseline subtraction. Full range of collagen concentrations was tested at 0.125°C/min heating rate. No dependence of the melting temperature on the concentration was found. Stable linear change of the temperature with time was observed at all heating rates. Slower than 0.004°C/min heating was prevented by thermogram distortions associated with slow temperature changes in the room.

Circular Dichroism. CD spectra from 210 to 250 nm were measured at room temperature in a J810 (Jasco, Easton, MD) spectropolarimeter equipped with a 150-W xenon lamp. To avoid protein damage, cumulative exposure of any sample to UV never exceeded 10 min.

For isothermal melting experiments, 0.8 mg/ml solutions of monomeric collagen were equilibrated in a theremoelectric temperature block (ThermoKool) continuously monitored by a digital thermistor thermometer (Cole–Palmer). Small aliquots were taken after predetermined time intervals, transferred to a quartz cell with 1-mm path length, and rapidly cooled to room temperature by nitrogen flow (to prevent further collagen denaturation or renaturation). The spectra were measured within 1–2 min after the transfer. Similar results were obtained when aliquots were discarded after each CD measurement and when the aliquots were returned into the sample for further equilibration. The fraction of native protein was evaluated from the change in ellipticity at 221 nm.

For isothermal refolding experiments, 0.8 mg/ml solutions of rat-tail-tendon collagen were denatured by 10-min equilibration at 55°C. The CD spectrum of each solution was measured at room temperature immediately before and after the denaturation as described above. Following the denaturation, the solutions were equilibrated in a temperature block at temperatures ranging from 5 to 30°C. The kinetics of triple helix refolding was followed using the same approach as for denaturation kinetics.

Comparison of Scanning and Isothermal Experiments. In principle, scanning measurements at a heating rate \( v_h \) should yield the same apparent melting temperature as a set of isothermal measurements at a fixed equilibration time. Indeed, if \( \Delta T \) is the width of the melting peak in the scanning experiment, then \( \Delta T / v_h \) is the time during which the protein melts in the process of the scanning experiment. Thus, if one were to equilibrate the protein at the temperature equal to the temperature at the maximum of the denaturation peak, the equilibration time for melting of 50% of the protein would be \( \Delta T / v_h \). To be more precise, this time should be \( \beta \Delta T / v_h \), where \( \beta \) is a numerical constant that depends—e.g., on the instrumentation parameters (thermal conductivity, cell volume, feedback loop power, etc.). The value of \( \beta \) can be found by matching scanning and isothermal data, as it is done in Fig. 2. For instance, the same DSC data for rat-tail-tendon collagen are plotted in Fig. 1 vs. heating rate and in Fig. 2 vs. equivalent equilibration time (open circles, \( \beta \approx 0.1 \)). The fastest DSC heating rate (0.004°C/min) corresponds to \( \approx 40 \) min equilibration.

Characterization of Refolded Collagen. After renaturation, collagen solutions were dialyzed against 0.5 M acetic acid (pH 2.7) and digested by 10 mg pepsin/1 mg collagen for 1 h at 35°C to cleave all partially renatured (gelatin) helices. Collagen (gelatin) was purified from pepsin by precipitation in 0.9 M NaCl/0.5 M acetic acid and subsequently redissolved in and extensively dialyzed against 2 mM HCl. The solution was mixed 1:1 with 0.26 M NaCl/60 mM sodium phosphate (pH 7.4 after mixing) to initiate fibrillogenesis and left overnight at 32°C. The fibers were spun down; the fibrillogenesis capable (pellet) and incapable (supernatant) fractions were redissolved (dialyzed) in 0.2 M sodium phosphate/0.5 M glycerol (pH 7.4) and used for DSC and SDS gel electrophoresis.

Temperature Validation. To validate absolute temperatures, all temperature controllers and thermometers were tested and (if necessary) calibrated with 0.1°C accuracy by using mercury ASTM thermometers (Fisher) certified by the manufacturer to meet National Institute of Standards and Technology specifications. The temperature inside the capillary cell of the DSC instrument was validated by a thin thermocouple wire, which was threaded through the cell.

Results

Equilibrium Collagen Denaturation Is an Ultra-Slow Process. To achieve much slower heating rates (or much longer equilibration times) than reported previously, we combined ultra-slow DSC and isothermal CD measurements. By using a modern DSC instrument we achieved the 0.004°C/min heating rate (Fig. 1), which corresponds to \( \approx 40 \) min equilibration (see Fig. 2). In isothermal CD, by adding enzyme inhibitors and taking precautions to avoid UV damage, we extended the equilibration time to \( \approx 10 \) days (Fig. 2a and b). But, we did not reach the
equilibrium. The dependence of the observed $T_m$ on the melting time (or heating rate) was still logarithmic even at the slowest measurement and could not be extrapolated to equilibrium (Fig. 2c). Apparently, the characteristic equilibrium denaturation time of collagen is much larger than 10 days. Perhaps it is months or even years. Such long equilibration was not practical—in e.g., because of collagen degradation noticeable on SDS/PAGE of sample aliquots despite the presence of protease inhibitors.

The Equilibrium State of Monomeric Type I Collagen at Body Temperature Is a Random Coil Rather than Helix. It follows from Fig. 2c that at 37°C type I collagen from human lungs converts to random coils within 2 to 3 days. Type I collagen from rat-tail tendon is even less stable. In contrast to the existing consensus, both proteins undoubtedly melt even several degrees below body temperature and their thermodynamically favored conformation at body temperature is a random coil. (CD spectra similar to those shown in Fig. 2a confirmed that the observed slow transition is complete melting and random coil formation.)

Refolding of Type I Collagen Triple Helix Occurs only at Temperatures Substantially Below Body Temperature. Because reversibility is probably the most important indication of the equilibration nature of protein unfolding, we investigated refolding of heat-denatured rat-tail-tendon collagen in the same buffer as we used for melting experiments. Using isothermal CD measurements, we observed rapid refolding (within several minutes) below 10°C. Above 10°C, the refolding time increased with temperature up to ∼10 days at 28°C (Fig. 3a and b). No refolding of rat-tail-tendon collagen was observed above 28°C. Although we observed 60% recovery of the triple helical CD signal (Fig. 3a), DSC thermograms of solutions renatured below 20°C were distinctly different from native collagen (Fig. 3c, blue line). No intact α1 and α2 chains were observed by SDS gel electrophoresis after pepsin cleavage and the solutions were incapable of forming fibers. As one would expect (11), only short-length gelatin helices formed at these conditions.

Slow in Vitro Refolding of Type I Collagen Produces a Mix of Full-Length α1(1)2α2(0), α1(0)2α2(1), and α2(1)2 Triple Helices. Refolding above 20°C (Fig. 3c, green and red lines) produced some full-length triple helices as indicated by DSC (Fig. 3c, cyan and magenta lines) and by gel electrophoresis after pepsin digestion (Fig. 3d). Pepsin-digested solutions contained a fraction of molecules that exhibited normal fibrillogenesis. This fraction consisted mostly of full-length α1(1)3 homotrimers and a much smaller amount of full-length α1(1)α2(2) heterotrimer (Fig. 3c, red line, and Fig. 3d, lane 1). Both of these are natural type I collagen species and both are known to exhibit efficient in vitro fibrillogenesis (12). Virtually all of these molecules should incorporate into fibers. Thus, the intact α1 and α2 bands in the fibrillogenesis-incapable fraction (Fig. 3d, lane 2) indicate that this fraction contains full-length α2(1)1 and α1(1)α2(1)1 helices in addition to pepsin-cleaved gelatin fragments (see also Fig. 3c, cyan line).

The abundance of α2(1)1 and α1(1)α2(1)1 molecules among full-length renatured helices indicates that α2 chains prefer to associate with each other rather than with α1 chains. Such interaction and formation of these helices are prevented in vivo by association of C-propeptides (13). Although formation of these unusual collagen species in vitro is an interesting phenomenon, it is beyond the scope of the present work.

Collagen folding involves many steps and many possible long living misfolded conformations (14). It is not surprising that only a small fraction of molecules were able to refold into full-length helices. Still, because such helices do form spontaneously, heat denaturation is a fully reversible process that can be characterized by an equilibrium $T_m$ although the true equilibrium melting is not achievable in practice because of its very long characteristic time.

Discussion

Thermal Denaturation of Type I Collagen Is a Fully Reversible, Intrinsically Equilibrium Process Accompanied by Large Hysteresis. It was recently proposed that collagen melting might be an irreversible rate-limited process that can be described by a first-order reaction kinetics and a simple Arrhenius temperature dependence (8, 15, 16). Although our denaturation data are in complete agreement with the corresponding data reported in these studies, we disagree with implicit interpretation of collagen denaturation as an irreversible (nonequilibrium) process. Indeed, our refolding data prove that thermal denaturation of type I collagen is a fully reversible process, as argued in refs. 14 and 17. We agree with the authors of the latter studies that that the apparent irreversibility of collagen denaturation at the time scale of several hours and the observed temperature dependence of the apparent $T_m$ are simply manifestations of the hysteresis characteristic for all cooperative equilibrium transitions.

Indeed, our data suggest that collagen is overheated by at least several degrees above equilibrium in all reported denaturation experiments, due to the extremely slow equilibrium kinetics. Denaturation of overheated collagen occurs much faster than
renaturation creating an appearance of an irreversible, rate-limited process the apparent $T_m$ of which depends logarithmically on the equilibration time. This dependence should break down and $T_m$ should become time-independent at the time scale comparable or larger than the equilibrium melting time. But, the latter appears to be so long that the logarithmic dependence is observed in the full range of time scales accessible to laboratory experiments. Therefore, extrapolation of the observed dependence cannot produce meaningful results.

There may be several reasons for the extremely slow kinetics of equilibrium denaturation and renaturation of collagen. For instance, this may be caused by a large activation energy (8, 15). But, the description of denaturation of this very large protein in terms of a simple first-order reaction kinetics with a single energy barrier may not be appropriate. Collagen denaturation/renaturation may also be extremely slow because complete unfolding/refolding of the helix may occur in many steps and via different pathways (14, 17). Unfortunately, the available data are not sufficient to distinguish these alternatives.

**Low Thermal Stability and Its Potential Roles in Metabolism and Function of Type I Collagen.** Thus, we can conclude that type I collagen is thermodynamically rather than kinetically stable. But, it is thermodynamically stable only below body temperature. The equilibrium $T_m$ of collagen monomers in physiological solution is several degrees below body temperature ($T_m < 36^\circ C$ for human lung and $28^\circ C < T_m < 35^\circ C$ for rat-tail-tendon collagen). The melting time at body temperature is from several hours (rats) to several days (humans)—i.e., it is comparable with the time from collagen folding to its incorporation into fibers (18–21). This observation may explain why a large fraction of newly synthesized collagen is degraded (19–21) and never makes it into mature fibers.

Why would Nature design this ubiquitous protein to be unstable at body temperature? How do cells manage to fold it if it is unstable? We cannot answer these questions unequivocally yet, but the following picture seems to emerge.

Apparently, procollagen is more thermally stable inside than outside cells (22). At least one collagen-specific endoplasmic reticulum chaperone (Hsp47) was found to stabilize collagen triple helix and to assist in its folding (23, 24). The exact role of Hsp47 in collagen metabolism is still being debated (13, 25). Nevertheless, it is likely that cells use Hsp47 and may be some other chaperones to fold the procollagen triple helix that would not form otherwise.

On secretion from cells procollagen molecules lose chaper-
ones. At that point they are likely to become unstable (our measurements indicate, for example, that neither propeptides nor extracellular matrix proteoglycans have any substantial effect on the triple helix stability). The molecules begin to unfold, probably starting from most temperature-labile domains (5). Such “microunfolding” was proposed to be essential for fibrillogenesis (26, 27) and, as indicated by our recent data (not shown), it appears to trigger the process after the cleavage of N- and C-propeptides. Fiber formation results in substantial stabilization of collagen helices (1) and prevents further unfolding. Defective and “lost” collagen molecules as well as molecules cut out by collagenases on matrix reconstruction should denature completely within several days. Then they become susceptible to common proteolytic enzymes allowing easy “clean-up.”

Nature adjusts thermal stability of collagen monomers to the body temperature in warm-blooded animals or to the environment temperature in cold-blooded animals by changing the hydroxyproline content of the protein (1). However, instead of adjusting temperature in cold-blooded animals by changing the body temperature in warm-blooded animals or to the environment temperature (1), Nature appears to adjust it to several degrees below body temperature. In addition to simplifying the clean-up, the other advantage of such design could be the following. Apparently, helices confined in fibers cannot melt completely because their confinement would not allow chains to gain as much entropy as in solution (15). Nevertheless, the molecules can melt and refold locally as needed (1), giving fibers their great combination of strength and elasticity.

In conclusion, changes in collagen stability—e.g., due to osteogenesis imperfecta mutations—have long been suspected to play an important role in disease (6). We can now look more closely at possible molecular mechanisms relating minor \( T_m \) changes to major connective tissue pathology caused by such mutations. For instance, a decrease in \( T_m \) by a little over 1°C changes the rate of collagen melting by an order of magnitude, reducing the unfolding time at the body temperature from several days to several hours (Fig. 2c). As a result, the fine balance between the kinetics of fiber formation and kinetics of collagen melting may be disrupted and fibers may have to form too fast. Their network may become less regular causing improper mineralization and brittle bones. Understanding the regulation of these processes may help us to find treatments for this devastating disorder. We hope that the present study is a significant step toward this goal.