

Evidence that Collagen Fibrils in Tendons Are Inhomogeneously Structured in a Tubelike Manner

Thomas Gutsman, Georg E. Fantner, Manuela Venturoni, Axel Ekani-Nkodo, James B. Thompson, Johannes H. Kindt, Daniel E. Morse,[†] Deborah Kuchnir Fygenon, and Paul K. Hansma

Department of Physics, University of California, Santa Barbara, California 93106, and the [†]Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California 93106

ABSTRACT The standard model for the structure of collagen in tendon is an ascending hierarchy of bundling. Collagen triple helices bundle into microfibrils, microfibrils bundle into subfibrils, and subfibrils bundle into fibrils, the basic structural unit of tendon. This model, developed primarily on the basis of x-ray diffraction results, is necessarily vague about the cross-sectional organization of fibrils and has led to the widespread assumption of laterally homogeneous closepacking. This assumption is inconsistent with data presented here. Using atomic force microscopy and micromanipulation, we observe how collagen fibrils from tendons behave mechanically as tubes. We conclude that the collagen fibril is an inhomogeneous structure composed of a relatively hard shell and a softer, less dense core.

INTRODUCTION

Collagens are a family of structural proteins reinforcing a variety of animal tissues including skin, bone, and tendon. Of all of the nonmineral constituents of the mammalian body there is more collagen than anything else. However, our understanding of the structure of collagen fibrils is rudimentary. Collagen diseases are typified by mechanical failure of associated tissues, and collagen products (leather, glue, gelatin) are valued for their unique mechanical properties. Knowledge of the molecular basis of the mechanical properties of collagen structures is thus important for both medical and technological applications.

In tissues, fibular collagens often form fibrils several hundred nanometers in diameter (Kadler, 1994). Native collagen fibrils exhibit a regular 67-nm banding observable in electron micrographs (Schmitt et al., 1942; Chapman and Hulmes, 1984). However, this D-spacing and banding pattern can also vary (Beniash et al., 2000). Banding also appears in topographical atomic force microscope images of hydrated, native fibrils (Fullwood et al., 1995; Bigi et al., 1997; Raspanti et al., 2001). X-ray diffraction data demonstrate that at least some collagen molecules within rat tail tendon fibrils have a well-defined crystal structure (Bear, 1942; North et al., 1954; Fraser et al., 1983; Fratzl et al., 1993; Hulmes et al., 1995; Prockop and Fertala, 1998). These data, however, also indicate that some, perhaps most, collagen molecules in rat tail tendons are in a state of liquid-like disorder (Fratzl et al., 1993; Hulmes et al., 1995; Prockop and Fertala, 1998). Moreover, nuclear magnetic resonance studies indicate that the collagen backbone is free to reorient within a collagen fibril (Trochia, 1982; Sarkar et al., 1985), and hydrated fibrils contain unbound liquid water

(Shinyashiki et al., 1990; Price et al., 1997). These facts, taken together, lead to several dilemmas. How can collagen fibrils be both ordered and disordered? Why do fibril surfaces appear stable in atomic force microscope images, if collagen can freely reorient within a fibril? Further, it has been described that the cross section of collagen fibrils contains central low density areas (Mallein-Gerin and Garrone, 1986). How can fibrils be homogeneously structured and contain areas of different density? In this paper we shed light on these questions with atomic force and light microscope data indicating that collagen fibrils act mechanically as tubes.

MATERIALS AND METHODS

Materials

Tails from rats sacrificed for other experiments were frozen (-20°C), typically for weeks, before our experiments. Rat tail tendons were removed from thawed rat tails and stored in phosphate-buffered saline solution (PBS, Sigma, St. Louis, MO) for several minutes before sample preparation.

AFM sample preparation and imaging

The atomic force microscope (AFM) image in Fig. 1 was taken from an epoxy (2-Ton Clear Epoxy, Devcon, Danvers, MA) mold of collagen fibrils. The mold was prepared by first placing $\sim 100\ \mu\text{l}$ of well-mixed epoxy on a steel disk. The disk was then heated to 60°C on a hot plate for several minutes. Heating resulted in a smooth and nearly cured epoxy surface. After removing the disk from the hot plate and cooling it down, a wet, disrupted tendon was spread over the epoxy. The tendon was then dried with a stream of dry air. The force of the air on the tendon during drying appears to press the tendon into the epoxy, resulting in a faithful mold of the collagen fibrils exposed on the disrupted tendon's surface. After drying, the tendon was removed with tweezers, and the epoxy surface was imaged with a commercial AFM (Multimode, Digital Instruments, Santa Barbara, CA) in contact mode using a commercial AFM cantilever (Contact Ultralever, Topometrix, Sunnyvale, CA). After imaging, the resulting topographical image was inverted to reconstruct the topography of the collagen fibrils that made the mold. This process allowed us to attain high-resolution images of a wide field of compressed collagen molecules. The molds originate from wet fibrils and can be scanned at a higher resolution than wet fibrils themselves.

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Address reprint requests to Thomas Gutsman, Tel.: 805-893-3999; Fax: 805-893-8315; E-mail: tguts@physics.ucsb.edu.

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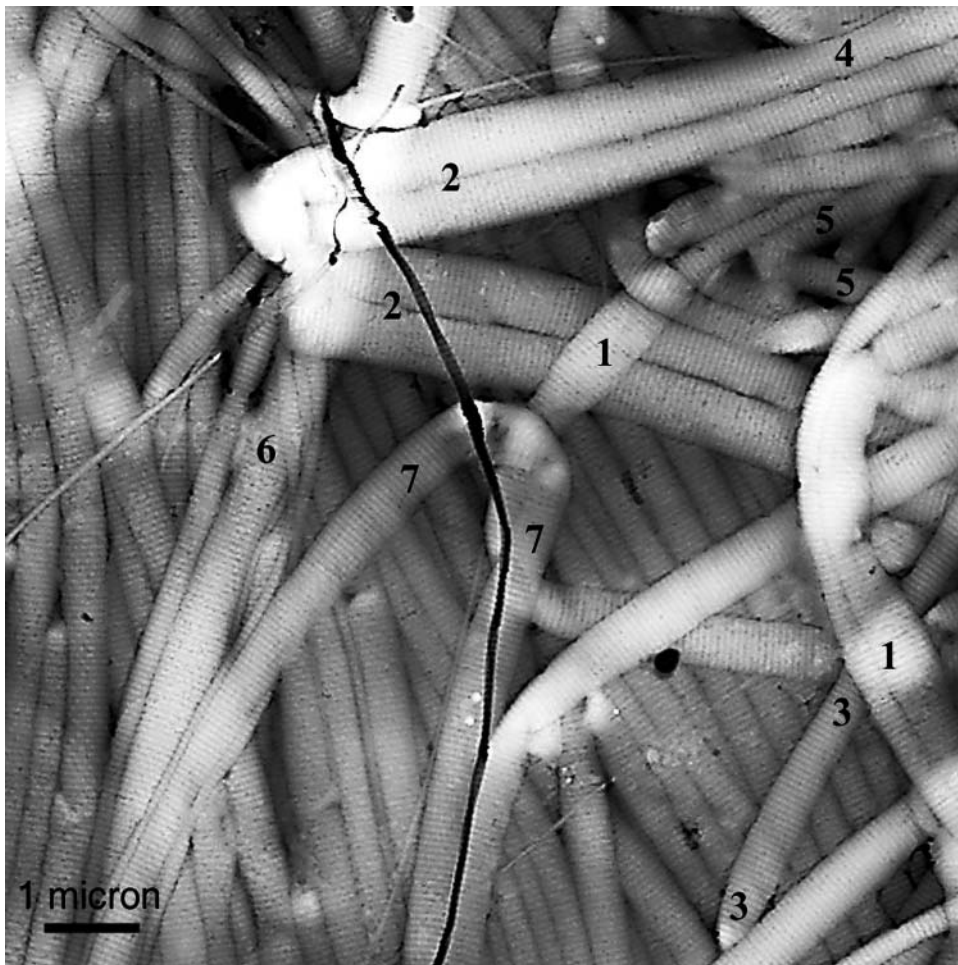


FIGURE 1 AFM image of an epoxy mold of compressed collagen fibrils. This replica AFM image illustrates the tubelike mechanical behavior of collagen fibrils. (1) The tubes flatten as they are compressed to go over underlying tubes. (2) When the tubes bend back on themselves there is no loop at the end as would have been expected for a solid, rod-like cylinder. The tubes can fold on themselves. (3) It is possible to see subtle structure from underlying tubes in flattened tubes (note region between the two 3s). (4) The tubes can be compressed laterally. (5) This tube and one above it fold over on themselves. (6) Flattened tubes can contour around other tubes. (7) The crumpling in this bend is characteristic of tubes, not of solid, rodlike cylinders.

Furthermore, by scanning a replica of the fibrils we avoid the problem that we might apply local mechanical forces on the fibril by the cantilever tip that is strong enough to distort the fibrils topography by compressing softer regions more than stiffer regions. The AFM images in Figs. 2 and 4 were taken from dried collagen fibrils of rat tail tendons which were prepared wet on a mica disk and dried with a stream of dry air. They were imaged in tapping mode using a commercial AFM cantilever (Tap300 (MPP-11100), Nanodevices, Santa Barbara, CA and NSC15 MikroMasch, Portland, OR).

Optical sample preparation and imaging

Two parallel rails supporting XYZ translation stages for micropipette positioning (SD Instruments, Grants Pass, OR) were mounted on each side of the stage of an inverted microscope (Olympus IX70, Melville, NY). An open sample was made by securing a 5-mm thick \times 42-mm diameter rubber o-ring to a 35 \times 50 mm coverglass (No. 1) with double-sided tape and sealing with wax. The cover slip was pretreated with 100 mM Mg-acetate (Aldrich, St. Louis, MO) to enhance fibril adhesion. Rat tails were thawed, their tendons removed, and thin fibers obtained by slicing lengthwise along the tendon. Collagen fibrils were spread in the center of the open sample and the o-ring was filled with PBS to prevent drying and minimize the effect of surface tension on the pipettes. Micropipettes were pulled from 1 mm borosilicate capillaries (Sutter Instrument Co., Novato, CA). Fibrils were imaged in brightfield using an air condenser (0.9 NA) and recorded live through a charge-coupled device (Dage-MTI, 640x480, Michigan City, IN) on a videotape recorder.

RESULTS

The atomic force microscope (AFM) (Binnig et al., 1986; Rugar and Hansma, 1990) has been used to image collagen molecules and collagen fibrils from a variety of sources (Fullwood et al., 1995; Raspanti et al., 2001; Chernoff and Chernoff, 1992; Revenko et al., 1994; Bigi et al., 1997; El Feninat et al., 1998; Miyagawa et al., 2000; Paige and Goh, 2001). We have used a molding technique to obtain images of compressed collagen fibrils from rat tail tendon. Besides the linear collagen fibrils showing the D-period of \sim 67 nm, which are well described in literature, we found a number of remarkable features of the rat tail tendon fibrils (see Fig. 1):

1. The fibrils flatten as they are compressed to go over underlying fibrils.
2. When the fibrils bend back on themselves they buckle instead of forming a loop.
3. It is possible to see subtle structure from underlying fibrils in flattened fibrils (note region between the two 3s).
4. The fibrils can be compressed laterally.
5. This fibril and one above it fold over on themselves.

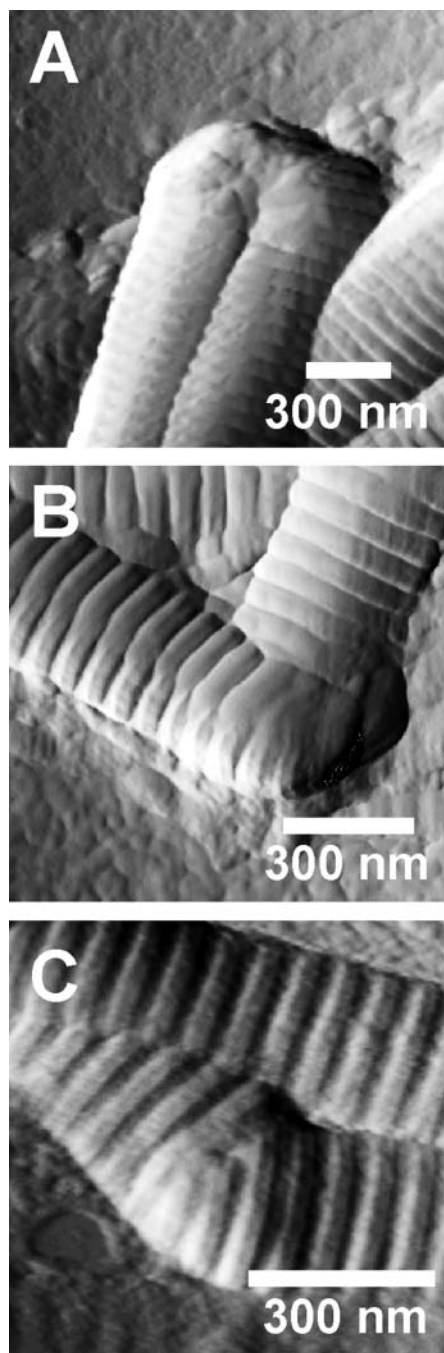


FIGURE 2 AFM images (amplitude signal) of dried collagen fibrils. Similar behavior is observed in this image of collagen fibrils as was observed in Fig. 1. These images show that banding continues right up to abrupt ends, suggesting that a banded, relatively rigid tube is fractured to expose a more flexible core material.

6. Flattened fibrils can contour around other fibrils.
7. At the tight curvature there is a crumpling of this fibril.

In Fig. 1 a single dark line can be seen through the middle of the image. This line is a collagen fibril that was not removed from the mold, but instead remained partially em-

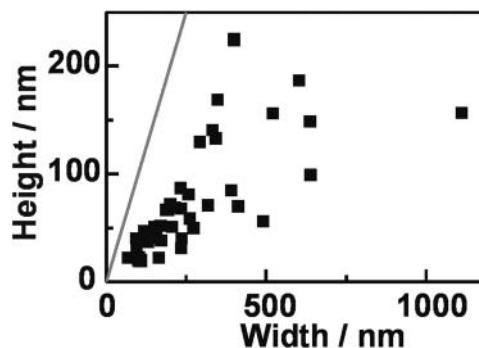


FIGURE 3 Correlation between width and height of dried rat tail tendon fibrils. The straight line represents a ratio of width to height of 1:1.

bedded in the epoxy. The banding on this fibril is similar in spacing and amplitude to that observed on the mold.

Images of dried (Fig. 2) and also of wet (data not shown) collagen fibrils show structures similar to those seen using the molding technique. At tight bends, collagen fibrils appear to lose their characteristic 67-nm banding (Fig. 2 *A*). Fig. 2 *B* shows a 90° kink of a collagen fibril demonstrating the break of an outer layer and the release of a softer unstructured inner material. Fig. 2 *C* also shows that a fibril did not bend with a continuous curvature but with a discrete angle while the D-period banding remains intact.

All single fibrils we found were flat and showed a ratio of width to height in average of 4 (Fig. 3). However, it was only possible to determine the accurate width and height from fibrils lying on the mica or glass surface separated from other fibrils and thus it is not possible to deduce a statistical distribution from the given values. In some images the section of a collagen fibril shows that the fibril is slightly higher at the edges than in the middle (Fig. 4).

To determine if this behavior is a more general principle of tendons we also investigated tendons from chicken legs with similar results (data not shown).

Images of dissected tendon taken under the light microscope show that fibrils can sustain sharp bends or kinks along their length. We have observed single fibrils buckle and kink under compression when manipulated with a micropipette (Fig. 5). Kinked fibrils become straight when no longer compressed, and have a tendency to kink again at lower compression and in the same location upon further manipulation.

DISCUSSION

The structure of collagen fibrils is one of the most intensively investigated structures of all proteins. However, there are still open questions. Therefore we did not focus on the very regular patterns of collagen fibrils but on uncommon properties of rat tail tendon fibrils.

The AFM and optical images provide evidence that fibrils from rat tail tendon behave mechanically like tubes rather

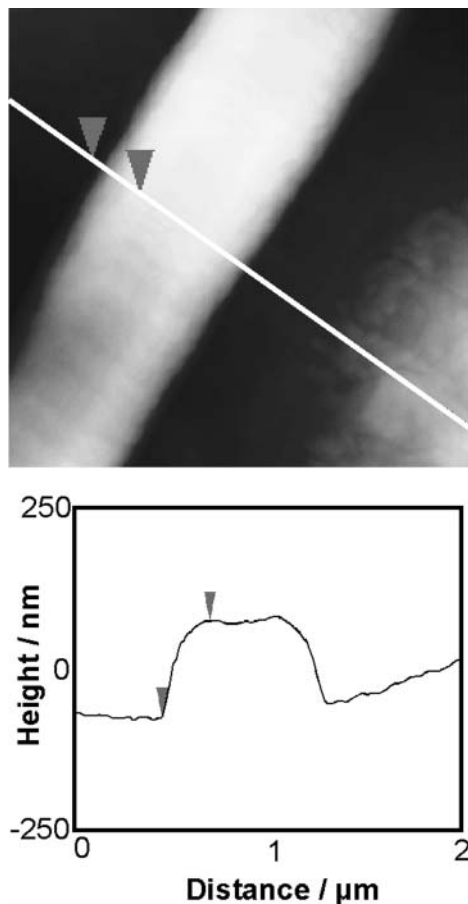


FIGURE 4 AFM image ($2 \times 2 \mu\text{m}$) and corresponding section analysis of a dried rat tail tendon. The cross section shows a ratio of width to height of ~ 5 and moreover the fibril is slightly higher at the edges than in the middle.

than rods. Further on we use the term “tube” not describing a hollow structure but a structure with different mechanical properties of the shell and the core, and the term “rod” for a homogeneously composed structure. For example, they can fold back onto themselves, make very tight turns or “kinks,” become flat as they are squeezed, and wrap around one another (Figs. 1 and 2). These are properties that are typical for a tube and not for a rod. This argues against the general assumption that collagen fibrils are in a homogeneously closepacked formation. Moreover the dried and even the wet fibrils appear flat when imaged by AFM on a glass substrate with a width to height ratio of ~ 4 (Fig. 3) showing that the overall structure of a fibril is rather soft. It has been shown that the innermolecular spacing of collagen decreases from ~ 1.6 to 1.1 nm upon drying (Hulmes et al., 1995; Price et al. 1997) which leads to a shrinking of the fibril’s volume. The width to height ratio of 4:1 is consistent with the assumption that fibrils are surrounded by a relatively hard shell which does not shrink as much as the softer inner material.

Our results imply that collagen fibrils have a mechanically inhomogeneous, tubelike structure. It is well known that

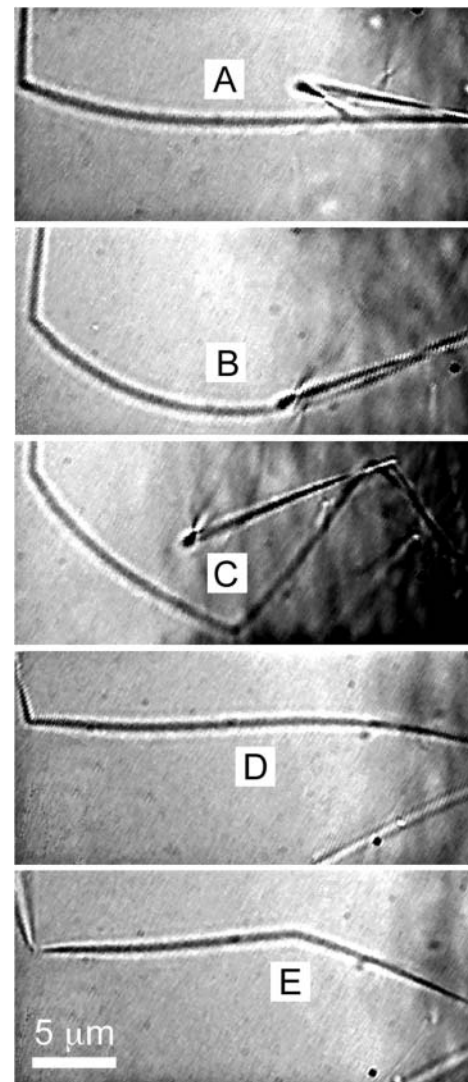


FIGURE 5 Video micrographs of collagen fibrils. (A) An individual fibril extends from a bundle off screen to an anchor point on the glass at the left of the frame. (B) The bundle is manipulated, causing the fibril to bend. (C) When the fibril is bent beyond a certain curvature, it forms a kink. (D and E) When the direction of bending reverses, the fibril snaps back to its original shape and eventually kinks in the opposite direction at the same point along the fibril.

covalent crosslinks exist between collagen molecules in tendon. Our data suggest that collagen molecules are more highly crosslinked near the fibril surface and more disordered or soft in the central region. This interpretation would explain why some, perhaps most, collagen molecules in rat-tail tendons appear to be in a state of liquidlike disorder based on x-ray diffraction data (Fratzl et al., 1993; Hulmes et al., 1995; Prockop and Fertala, 1998). It has been shown for many polymers that they can form intermixed crystalline and amorphous structures. It would also explain nuclear magnetic resonance data indicating that the collagen backbone is free to reorient within a fibril (Trochia, 1982; Sarkar et al., 1985), and hydrated fibrils contain unbound liquid water

(Shinyashiki et al., 1990; Price et al., 1997). It is consistent with structural hints in the literature of a distinct axial domain within collagen fibrils (Franc, 1993).

Our data give an idea about the supramolecular structure of rat tail tendon fibrils, however, we can not draw conclusions on the molecular organization of collagen proteins and other proteins being involved in the fibril formation such as proteoglycans (Scott, 1991). It is likely that proteoglycans, especially, have an important influence on mechanical properties of the fibrils.

We have not investigated the age dependence of the tubelike behavior. It is possible that the relatively rigid shell is a product of continuing polymerization by free radicals or enzymes in the fibrils of our mature animals. This would be consistent with the observation that fibrils grow larger with age (Franc, 1993).

Collagen has been strongly implicated in the toughness of bone (Zioupos et al., 1999). It has been proposed that fluidity within fibrils allows collagen molecules to slip relative to one another and thereby distribute stress and dissipate energy (Sarkar et al., 1987). It may be that such slippage under load allows the rupture of the self-healing sacrificial bonds (Smith et al., 1999), which have been shown to exist in collagen (Thompson et al., 2001). The rupture of such bonds would then dissipate energy and protect tendons from permanent damage. We propose that, in case of a high force, the stiff outer shell of the collagen fibrils could break while the fluid core remains intact and might be used in the repair of the shell. The results also suggest the possibility of transport of small molecules within and along tendons in vivo.

Why is a tube favorable instead of a rod? We can only speculate at this point. In the longitudinal direction the properties of a tube and a rod are comparable. However, in the transverse direction they have different characteristics. In the body of mammals the tendons often bend over other structures like bone and especially in this case the properties of a tube might be better than a rod. As shown in Fig. 5, a kinked fibril snaps back very easily and probably much faster into a linear conformation than a rod. Furthermore we might suggest that, when a bundle of rods bends over a structure the outermost filaments have to stretch much more than the inner ones. However, assuming that the mass of an internal, fluid substance can be displaced longitudinally, a bundle of tubes can squeeze together leading to a smaller ratio between the lengths of the opposite sides of the fibrils thus minimizing the deformation of the side away from the structure. This could preserve bonds that give the fibril strength and toughness.

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