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Investigations into the polymorphism of rat tail tendon fibrils using atomic force microscopy

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Abstract

Collagen type I displays a typical banding periodicity of 67 nm when visualized by atomic force or transmission electron microscopy imaging. We have investigated collagen fibers extracted from rat tail tendons using atomic force microscopy, under different ionic and pH conditions. The majority of the fibers reproduce the typical wavy structure with 67 nm spacing and a height difference between the peak and the grooves of at least 5 nm. However, we were also able to individuate two other banding patterns with 23 ± 2 nm and 210 ± 15 nm periodicities. The small pattern showed height differences of about 2 nm, whereas the large pattern seems to be a superposition of the 67 nm periodicity showing height differences of about 20 nm. Furthermore, we could show that at pH values of 3 and below the fibril structure gets dissolved whereas high concentrations of NaCl and $CaCl_2$ could prevent this effect.

Keywords: Collagen; Tendon; Fibril; Polymorphism; Atomic force microscopy; D-spacing

Collagen is an ubiquitous protein within connective tissues. There are at least 20 genetically distinct types of collagen [1]. Type I collagen the most abundant form of collagen (about 90%), is present in most tissues, primarily bone, tendon, and skin. It is the most regular one and most structure research has been done on this collagen type. The intimate nature of type I collagen packing interactions is, as yet, a subject of conjecture.

Since the invention of atomic force microscopy (AFM) in 1986 [2], it has rapidly become a widely used technique for characterizing biological macromolecules [3,4]. It provides a high resolution, three-dimensional surface image of a sample, which makes atomic force microscopy well suited for the investigation of the topology of biological macrostructures.

Type I collagen fibrils, in their native form, typically display a banding pattern of 67 nm spacing when visualized with transmission electron microscopy (TEM) [5,6] or AFM [7,8]. This spacing is called D-period. The significance and the origin of the 67 nm banding is still a source of discussion. Petruska and Hodge developed a widely accepted model of the organization of collagen molecules into fibrils, based on the results they obtained from TEM micrographs of negatively stained samples [9].

Three of the left-handed coiled collagen molecules form a right-handed coiled triple helix, the so-called tropocollagen [10]. For the structural organization of tropocollagen there are two different classes under discussion in the literature, the axial (longitudinal), and the equatorial (lateral) packing in the fibrils. Smith first proposed a subfibrillar model of a cylindrical filament built from five tropocollagens [11]. A different model is the quasi-hexagonal molecular packing model with molecules inclined at 5.2° to the fibrils axis [12]. Combination of these two models led to the model of the compressed microfibrils [13]. It is well accepted that the assembly of collagen molecules into fibrils is an entropy-driven process [14]. However, all different models are not consistent with all data obtained from different techniques.

Using atomic force microscopy, we were able to obtain further information regarding the hierarchical

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structure of collagen. Recently, we observed that collagen fibrils do not act mechanically as rods but as tubes [15], indicating the inhomogeneity of the molecular organization of collagen into fibrils. These fibrils consist of a stiff outside surface (the shell) and a soft core. Chen et al. [16] have already proposed these properties. In this paper, we are focusing on the topological profile of the stiff outside surface.

Lin and Goh [17] showed that height corrugation in AFM corresponds to the regions of high and low absorption observed in TEM imaging. TEM investigations have recently described the presence of small banding on collagen fibrils derived from the distal zone of the collagenous matrix of vitrified ice sections of dentin prior to mineralization [18]. This small periodicity has been quantified to 23.5 nm. When evaluating the model of molecular organization of collagen molecules, one has to consider all of the different polymorphisms of collagen.

Therefore, we characterized different spacing patterns with the AFM and investigated the effect of pH and ions on the fibril structure and integrity.

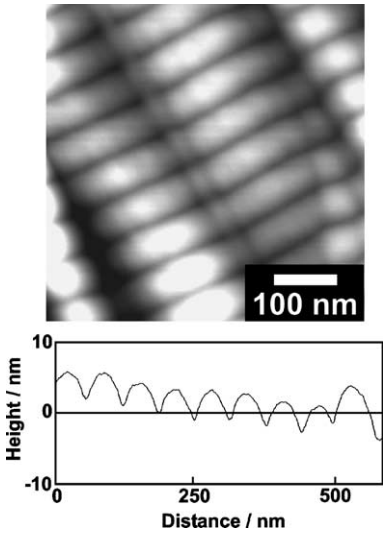

Fig. 1. AFM image and sectional analyses of rat tail tendon collagen type I fibrils showing the typical 67 nm periodicity. Top: height image taken in non-contact mode, bottom: longitudinal section of a fibril.

Materials and methods

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

The images in Fig. 5 were taken from collagen fibrils prepared in deionized water at pH 3 the first without and the second with 500 mM $CaCl_2$. Images in Figs. 6 and 7 originate from samples that were initially stored overnight in 500 mM NaCl, 1 mM citric acid, 1 mM Tris resulting in a pH of 3. Afterwards, the solution was brought to a physiological pH of 7.4. The sample was then imaged after a few hours.

Fibrils from rat tail tendons were prepared wet on a mica disk and dried with a stream of filtered air. They were then imaged with an AFM (Veeco/Digital Instruments, Santa Barbara, CA, USA) in contact and non-contact mode using commercial cantilevers (Tap300 (MPP-11100), Nanodevices, Santa Barbara, CA, USA and CSC12 or NSC15, MikroMasch, Portland, OR, USA).

Results and discussion

In order to get new information about the structural organization of collagen molecules in tendons, we imaged rat tail tendon collagen fibrils under different conditions, focusing on deviations from the standard appearance. Initially, we characterized the depth of the 67 nm banding. As can be seen in Fig. 1, the height difference between the peaks and the grooves are at least 5 nm, as reported earlier [17]. When mapping topography of biological samples using AFM, one has to take into account the tip-sample interactions and the deformations that may occur. To establish that the corrugation is a topological effect rather than a manifestation of areas of different densities, we changed the imaging force and performed contact and non-contact imaging. We also used a molding technique to obtain an imprint that has the same topography as the collagen fibril but homogenous density of the mould resin (data not shown). The local pressure applied on the sample, while making the mold, is much less than the pressure generated by the tip of the AFM when scanning. All attempts led to height differences ranging from 3 to 7 nm. Because of the finite radius of cantilever tips, it needs to be considered that the grooves observed by AFM imaging may be underestimated [19,20]. Katsura and Ono also showed height differences in TEM images of surface replica [21] and samples of wet collagen fibrils [22]. The density differences revealed in TEM could also be attributed to topography due to the different deposition of the stain in the grooves and on the peaks, as shown by Paige et al. [17,23], which would be consistent with what is observed in AFM imaging. Also other molecules are involved in the fibril formation such as proteoglycan [24], and one might argue that the grooves are influenced by proteoglycans. However, the height difference increased after removing the proteoglycans from the collagen of human sclera [25,26]. All of these measurements led to a height corrugation of 3–7 nm, which cannot easily be explained by the overlap mismatch of single triple helices having a diameter of about 1.6 nm [27,28] as described by the model proposed by Petruska and Hodge [9].

We also found a smaller banding with a periodicity of about 23 ± 2 nm, in addition to the standard 67 nm banding, in rat tail tendon collagen fibrils, which were prepared in 150 mM NaCl, 2 mM Tris at pH 7.4 or in PBS buffer (Figs. 2 and 3). The smaller banding,

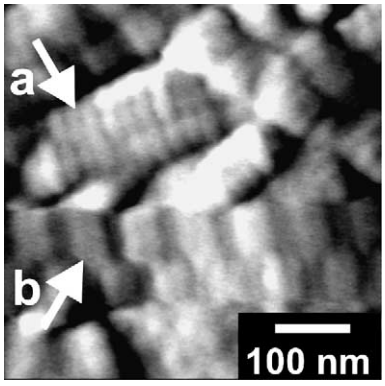

Fig. 2. Deflection image of a bundle of dried collagen fibrils, showing (a) a collagen fibril having a periodicity of about 23 nm and (b) a fibril with a typical spacing of about 67 nm.

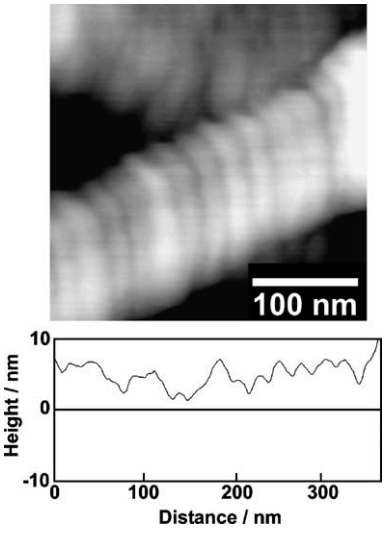

Fig. 3. AFM image and sectional analyses of rat tail tendon collagen type I fibrils showing a 23 nm periodicity. Top: height image taken in contact mode, bottom: longitudinal section of a fibril showing a spacing of about 23 nm especially on the right side.

observed in less than 3% of the images, occurred in samples prepared similar to the samples showing only the 67 nm banding. The height difference between the peaks and the grooves of the small banding was about 2 nm (Fig. 3). To our knowledge, this is the first time that 23 nm banding is described by AFM imaging. We did not find any correlation between the presence of the small banding and any particular sample preparation condition. Beniash et al. also observed this pattern by TEM and attributed it to either a lower level of organization of the fibrils or the association to different (specific) non-collagenous proteins. Osmotic pressure and removal or addition of highly anionic macromolecules was also indicated as a possible factor to play a role in the modification of the banding pattern observed in collagen fibrils [18]. Our data demonstrate that fibrils of the same bundle can show the 67 nm or the 23 nm

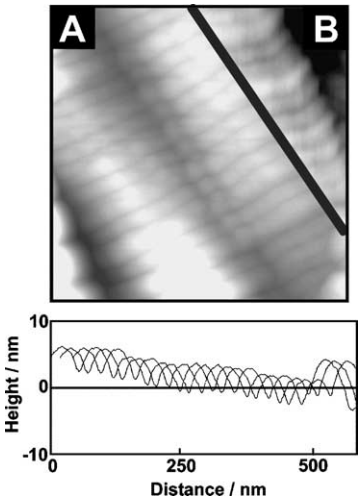

Fig. 4. Comparison between a three times overlay of the 67 nm periodicity with a displacement of 1/3D and the 23 nm periodicity. Top: (A) threefold overlay of Fig. 1 with a displacement of 1/3D along the axis of the fibril and (B) part of Fig. 3 showing a 23 nm periodicity. Bottom: threefold overlay of the longitudinal section of a fibril.

periodicity. A suitable model for the molecular organization of collagen should account for both the 67 and 23 nm periodicity. The 23 nm banding is about 1/3 of the D-spacing and one might suggest that this banding results from a 1/3 D-displacement of adjacent structural elements. To investigate this hypothesis we made a threefold overlay of Fig. 1 with a displacement of 1/3 D along the axis of the fibril and compared this result with Fig. 3 showing the 23 nm banding. Both figures show the same topography (Fig. 4, top). A threefold overlay of the longitudinal section of a fibril (Fig. 4, bottom) shows that the height difference between peaks and grooves should be small for the resulting 23 nm banding and in fact we observed this behavior. This is, however, not a proof of our hypothesis but a model to work on. The proposed displacement of structural elements should be on the lower level of the hierarchy of the tendon, e.g., tropocollagen or subfibrils. The basis of the formation of the 23 nm banding is not clear yet. Beniash et al. [18] propose that different banding patterns result from structural changes during the process of assembly and/or the binding of ions and macromolecules to the collagen.

To get further information on the organization of collagen molecules, we prepared rat tail tendons in deionized water at different pH values. Below pH 4, the collagen fibrils dissolved, which can be seen in Fig. 5A, showing a collagen fibril dissolved at pH 3. The presence of salt ions, e.g. Na^+ and Ca^{2+}, can prevent the fibrils from being dissolved in low pH conditions. At a $CaCl_2$ concentration of 500 mM at pH 3, the fibrils stayed intact and did not change their banding pattern (Fig. 5B). At a concentration of 500 mM of NaCl at pH 3, the organization of fibrils was preserved, but we observed a significant reduction in the height differences between

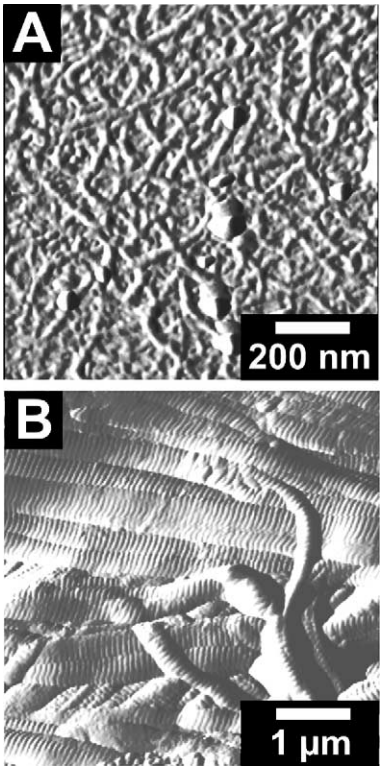

Fig. 5. Amplitude image of rat tail tendon collagen showing the decomposition of the fibrils at low pH and the protection from this effect induced by Ca^{2+}. (A) Collagen prepared in deionized water at pH 3; (B) collagen prepared in deionized water, 500 mM $CaCl_2$ at pH 3.

peaks and grooves of the banding pattern (data not shown). Collagen that was initially exposed to a pH of 3 and 500 mM NaCl was then brought to a pH of 7.4 prior to its preparation for AFM imaging. In this sample, we observed a periodicity of about 210 ± 15 nm superimposing the normal 67 nm banding (Figs. 6 and 7). The periodicity of 210 nm banding with a groove to peak distance of about 20 nm corresponds to the values obtained by Paige et al. [23] who described the "fibrous long spacing" (FLS) collagen type B. FLS has been described to occur both in vitro and in vivo. In particular, FLS collagen has been found both in pathological and normal tissues [29–33]. Two major hypotheses have been proposed about the origin of FLS in vivo. The first hypothesis suggests that FLS is the result of partial degradation of collagen reticular fibrils by endogenous collagenase [34]. The second hypothesis proposes that FLS is derived from the association of immature collagen microfibrils and acid mucopolysaccharides present in tumoral tissues [35]. Both of the hypotheses described above link the occurrence of FLS collagen to different physiological circumstances during assembly. We have shown, however, that also environmental conditions can change the structure of the assembly. It is remarkable that the fibrils show the 67 as well as the 210 nm spacing. Regarding the underlying structural organization of this pattern we can only speculate. It seems to be possible

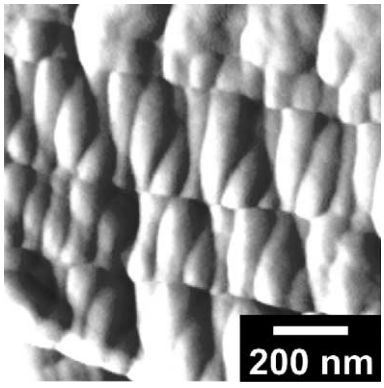

Fig. 6. Deflection image of a bundle of dried collagen (prepared at pH 3 with 500 mM NaCl then brought to pH 7.4 prior to imaging) fibrils showing the 67 nm periodicity and a superimposed periodicity of about 210 nm.

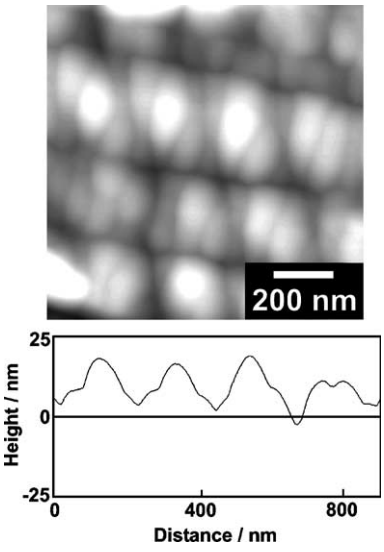

Fig. 7. AFM image and sectional analyses of rat tail tendon collagen type I fibrils showing an overlaying periodicity of about 210 nm. Top: height image taken in contact mode, bottom: longitudinal section of a fibril. The highest distance between peaks and bottom of grooves is quantified around 18 nm.

that the pH-shift lead to a swelling of parts of the banding pattern or even that a partial reorganization of the tropocollagen into an FLS-like structure occurred. Our data confirms that ionic interactions play an important role in the organization and the preservation of collagen structure, which was previously shown by Meek et al. [36] and Thompson et al. [37] using force spectroscopy experiments on collagen molecules.

We have shown that environmental factors influence the structural appearance of collagen fibrils. A model describing the molecular organization of collagen in tendons should account for these polymorphisms, e.g. the 23 nm banding, the 210 nm superposition, as well as the 67 nm banding. In the past two different models for the arrangement of collagen molecules in a crystalline array have been proposed: (a) sheets of collagen mole-

cules, where molecular translations between segments are linear and (b) microfibrils of collagen molecules, where molecular translations are cyclic [12,38]. Wess et al. [39] showed in X-ray diffraction experiments that a 1D staggered left-handed microfibril structure is more likely. Following this model, we propose that the different spacings we observed originate from a change in the structural organization of the microfibrils. This change can be a ''misalignment'' of the cross-links between microfibrils. An enzymatic conversion of lysine and hydroxylysine residues into aldehyde groups leads to the cross-links, which are thought to give more stability to collagen structure, and whose formation and localization might be influenced by ions, pH or other molecules [40–42].

To provide a better model for the wavy structure of collagen fibrils Katsura and Ono introduced the helical filament model [21]. However, it is not easy to explain the 23 nm spacing with this model except if the helical filament could adopt different pitches.

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