Organization of hydroxyapatite crystals within collagen fibrils

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Transmission electron micrographs of individual mineralized collagen fibrils show that hydroxyapatite crystals are located mainly within the fibrils at the level of the gap regions. The plate-shaped crystals are observed to be more or less uniformly stacked across the fibril diameter. We therefore suggest that the crystals are primarily located in 'grooves' created by contiguous adjacent gaps. The proposal is consistent with the observed crystal distribution in the fibril and with their average widths, which are almost 10-times greater than an individual gap diameter.

Collagen *Hydroxyapatite crystal* *Biomineralization* *(Bone, Turkey tendon)*

1. INTRODUCTION

Ultrastructural studies of the collagen and hydroxyapatite crystals in bone show that they are intimately related. This is particularly well demonstrated in transmission electron micrographs (TEM) of unstained thin sections of newly formed bone which show the characteristic collagen repeat structure [1,2]. X-ray [3] and electron diffraction [1] patterns of localized areas in bone also show that the crystals and collagen fibrils are spatially related, in that the hydroxyapatite *c* axes are well aligned with the collagen fiber direction and the crystals are located at the level of the gap regions [4,5]. The details of this relation are, however, poorly understood.

The technical difficulties in studying the molecular organization of bone are manifest. The crystals themselves are exceedingly small [4,5], and the type I collagen fibrils have a large range of diameters and are generally densely packed. The result is that even the thinnest of sections prepared for the TEM abounds with fibrils and contains a multitude of crystals. In this study we alleviate these technical difficulties by studying individually mineralized collagen fibrils in the TEM. We take advantage of a chance observation that sonication of calcified turkey tendon fibrils results in their desegregation into particles, some of which are individual fibrils.

Calcified turkey tendons are only partially analogous to bone. In mature bone the mineral is always associated with the collagen fibrils, but is also very often present between fibrils [6–8]. Calcified turkey tendons, on the other hand, are composed almost entirely of close-packed mineralized fibrils [9], with little room between fibrils. Their ultrastructure and stages of mineralization have been well studied by histology [10], electron microscopy [9,11], X-ray, electron and neutron diffraction [11–13]. In all published studies whole tendons or embedded and thin-sectioned material were used. Here we report the results of a TEM study of individual mineralized collagen fibrils, show that the crystals are located in a regularly ordered manner within the collagen

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fibril, and suggest that the primary location for the crystals is within the grooves formed by contiguous adjacent gaps.

2. MATERIALS AND METHODS

Fresh calcified leg tendons were obtained from a 21-week-old turkey and frozen until use at −4°C. The centrally located mineralized portion was finely shredded by scraping lightly with a scalpel. The shreddings were placed in a capped microcentrifuge vial containing double-distilled water or spectroscopically pure ethanol and then sonicated for 45 min. One drop of the suspension was placed on a carbon-coated EM grid. After 15 s the grid was placed on a clean microscope slide precooled in liquid nitrogen vapor. After freezing, the slide was rapidly transferred to a vacuum desiccator and

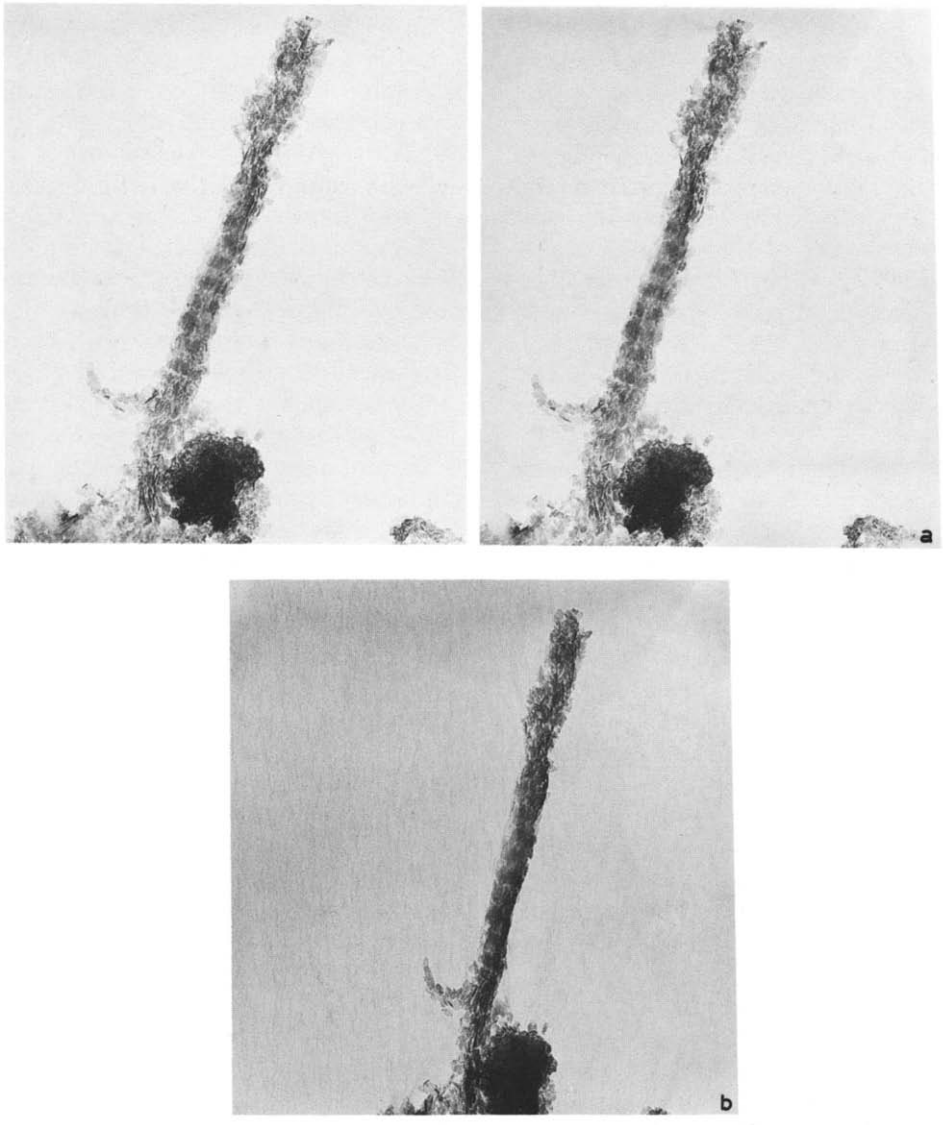

Fig.1. (a) Stereoview of an unstained mineralized collagen fibril at 0° tilt. The frothy appearance of the crystals suggests that they have suffered some radiation damage. (b) The same fibril tilted by 45° about its long axis. Magnification 46000 ×.

evacuated overnight. The sample was removed from the desiccator just prior to insertion into the Philips 400 TEM for examination. Samples embedded in epon and sectioned transversely across the forming ends of a tendon were also examined.

3. RESULTS

Fig.1a shows a stereoview of an individual mineralized fibril, which in part shows the characteristic collagen banding pattern. The preparation is unstained and the observed density is almost entirely due to the hydroxyapatite crystals. The stereoview of fig.1a shows unequivocally that most of the crystals are within the fibril and are remarkably well ordered in its central section. Their presence within the fibrils was confirmed from stereoviews of transverse fibril sections (fig.2) showing crystals throughout their cross-section. Electron diffraction patterns of such sections show that the *c* axes of hydroxyapatite crystals, and hence the collagen fibril axes (see below), are oriented approximately perpendicular to these sections.

Individual hydroxyapatite crystals can be clearly seen in fig.1 when viewed in stereo. All the crystals have the same basic shape: thin, elongated, but irregularly shaped platelets. In the region which shows the collagen banding pattern, the longer dimensions of the crystals tend to be aligned with the fibril axis. Electron diffraction from this area confirms the well known fact that the *c* axes of the crystals are aligned with the fibril axis. Presumably then the crystals themselves tend to be elongated in the *c* axis direction. The crystals are all plate-like in shape and only appear as needles when the plates are viewed on edge. The 45° tilted view of the fibril (fig.1b) shown in fig.1a demonstrates this phenomenon.

Isolated crystals outside the fibril can also be seen in fig.1. The lengths and widths of 83 such crystals were measured from many different micrographs and found to be 334 ± 106 and 185 ± 63 Å, respectively. Measurements of 70 such crystals from fibrils that were dispersed in 100% ethanol showed the average lengths to be 290 ± 106 Å and average widths 165 ± 54 Å. The differences in average dimensions measured between the two preparations are probably not significant as the sample size is rather small. These results are consistent with those reported by Weiner and Price [5] for crystals extracted from calcified turkey tendon treated with sodium hypochlorite.

Repeat distances of the banding pattern as seen in fig.1 were measured from 5 different fibrils and found to be 623 ± 20 Å. This is less than is found in fresh collagen [14] and even slightly less than is

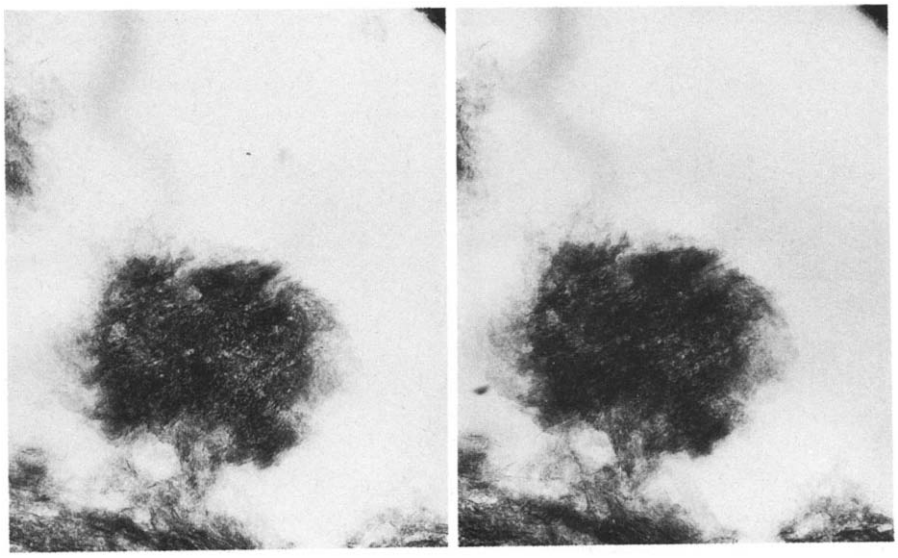

Fig.2. Stereoview of a transverse unstained section of an embedded fibril. Magnification 69000 ×.

found in embedded and sectioned material where the fibril is known to undergo contraction [15]. The specimens in this study have, therefore, suffered considerable contraction along the fiber axis. We therefore cannot make any statement as to whether crystals in vivo are confined to the gap regions, or as is quite apparent from fig.1a also extend into the overlap region.

One interesting observation is that the large majority of individual fibrils we observed were oriented on the grid such that the crystal plates within the fibrils were aligned more or less perpendicular to the beam. Fig.1a is a typical example. The simplest explanation is that the fibrils in aqueous suspension are not round in cross-section, but tend to be oval shaped. They settle onto the grid with their largest diameters in the plane of the grid. The cross-sectional oval shape is clearly demonstrated in the TEM; cf. fibril diameters of fig.1a at zero tilt and fig.1b at 45° tilt. This observation on its own could be attributed to a drying artefact. The fact that the crystals within the fibril are also aligned in the plane of the film suggests that this is not the explanation, although drying might well exaggerate the phenomenon. Independent supporting evidence for the crystals in the fibril having a preferred orientation across the fibril is shown in fig.3. This mineralized collagen fibril is twisted such that the crystals can be seen edge on, as well as approximately perpendicular to the plane of the plates. The edge on view clearly shows that the crystal plates are all aligned across the fibril. In this position, the observed arrangement is unlikely to be an artefact due entirely to the drying process. The fibril cross-section in fig.2 also shows preferred orientation of the crystals across the fibril.

Fig.3. Portion of an unstained twisted collagen fibril. The broader region near the center shows crystal plates lying approximately flat; above and below this region they are seen more edge on and appear as parallel dark lines. Magnification: 43000 ×.

4. DISCUSSION

This study confirms three previously recognized, but still disputed, properties of mineralized collagen fibrils: (i) crystals are located within the fibril, and presumably on its surface; (ii) the observed crystals are all thin, irregularly shaped plates; and (iii) a large number and possibly even all the crystals are in the gap or hole regions. The study also shows that the crystal plates are stacked more or less uniformly across the collagen fibril.

We show unequivocally that in calcified turkey tendon the crystals are located within the fibrils and not just on their surface. This is consistent with earlier observations [2]. The observation that the crystals seen in tendon are plate-shaped reaffirms Robinson's [16] original observation on human bone. Weiner and Price [5] recently came to the same conclusion for 6 different bones. The dimensions measured by Weiner and Price [5] and confirmed here for turkey tendon, using both hydrous and anhydrous preparation regimes, raise serious problems as to the locations of the crystals within the fibrils. Whereas the crystal lengths and thicknesses are consistent with their being located in the collagen gap regions, their widths are almost an order of magnitude larger than an individual gap diameter. The concept of one or more crystals

being located in a single gap is therefore not tenable [5]. A number of models of collagen fibril organization (e.g. [17]), including that first proposed by Katz and Li [18], predict that adjacent gaps are in contact with each other at the same 'height' in the fibril. The consequence is that extended grooves are formed whose lengths and thicknesses are the same as individual gaps, but whose widths are much larger. Hulmes et al. [19,20] directly observed such grooves in non-mineralized rat tail tendon fibrils. Weiner and Price [5] speculated that the crystals form in these grooves. The widths of the crystals are compatible with the dimensions of the larger grooves observed by Hulmes et al. [20]. The collagen models [17,18] predict that in the fibril cross-section the grooves should be arranged in parallel rows, as observed by Hulmes et al. [20] for local laterally crystalline regions. Our observations that the crystal platelets are stacked across the fibril are, therefore, consistent with the notion that they are located in the grooves.

All studies of this type are artefact prone and need to be carefully evaluated. The aqueous environment is conducive to preserving collagen fibril structure. The fibril samples prepared in 100% ethanol did not show well preserved structures. Drying, even by lyophilization, undoubtedly causes artefacts due to contraction. Thus the observed arrangements of crystals within the fibril must still be regarded as tentative.

Calcified turkey tendon is not homologous to bone. It can be thought of as comprising just one of the two 'compartments' in which mineral is found in bone [21], namely within fibrils and not between fibrils. It is, therefore, most likely that the observations reported here for calcified turkey tendon are also relevant to the intrafibrillar mineralization processes in bone.

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