X-ray diffraction of rat tail tendon at ambient and cryo-cooled temperatures - a comparison

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Fibre Diffraction Review **11**, 119-122, 2003

Introduction

Rat-tail tendon is primarily composed of type I collagen fibres in which the collagen molecules are axially packed according to the quarter-stagger model (Hodge and Petruska (1963). This arrangement produces the characteristic meridional series of reflections in the X-ray diffraction pattern of this tissue. Crystalline-like lateral packing interactions produce a series of discrete but often overlapping Bragg reflections arranged as rowlines in the equatorial plane of the X-ray diffraction pattern. Analysis of the equatorial reflections reveals that the collagen molecules are packed on a non-standard quasi-hexagonal lattice, where the unit cell is triclinic (Fraser *et al*.,1983; 1987; Wess *et al*., 1995; 1998; Orgel *et al*., 2001). The equatorial row-lines are overlaid by a continuous layer of diffuse scatter, thought to arise from liquid-like molecular disorder, particularly in the gap region (Hulmes *et al*., 1995). Some of the diffuse scatter is removed on cryo-cooling the sample to 90-100 Kelvin during data collection and some non-physically by mathematical means (Wess *et al*., 1998). Analysis of the component underlying Bragg peaks and of the distribution and intensity of the diffuse scatter at different temperatures will help to further clarify the positions, mobilities and paths of the molecular segments within the gap and overlap regions of the triclinic unit cell.

Materials and Methods

Medium angle X-ray diffraction of whole rat tail tendon in the cryo-cooled and ambient states was performed at stations 7.2 and 14.1, at the SRS Laboratory in Daresbury (UK). Whole tendons were mounted and decrimped in a customised cell and cryo-cooled in a liquid nitrogen gas shroud (Oxford Glycosystems™) to between 97.5 and 100 Kelvin (-176 to -173 °C; cooling rate set to experimental maximum), using 20 percent (v/v) glycerol in PBS, pH 7.4 as cryoprotectant. Some of the background diffuse scatter remaining after cryo-cooling was removed using in-house (CEMB) software. In this routine, a window of user-defined size roved across the image, ranking intensities and plotting the median as a background function, which was then subtracted from the original data. The background and subtracted original datasets were scaled and analysed using Fit2d (Hammersley, 1993) and Excel. TEM cryosections were prepared by colleagues at the University of Lausanne, Switzerland. For fibrils, rat tail tendon was crushed under liquid nitrogen and suspended in PBS (pH 7.4). A suspension of fibrillar collagen prepared in this way was flash-frozen to 133 Kelvin (-140°C) using 20 percent (w/v) dextrose as cryoprotectant and sectioned to 50 nm thickness. Images were analysed using Fit2d (Hammersley, 1993) and one-dimensional electron density line profiles were averaged and outputted using Excel. Fourier analyses to the 12th order were performed on each averaged line profile using in-house software.

Results and Discussion

(a) Cryocooling

Cryo-cooling the tendon prior to and during data collection altered the profile of the diffuse scatter in the near equatorial region of the diffraction pattern (Figures 1 and 2). This effect was possible without dehydration, and therefore without structural modification, of the sample. Cryo-cooling effectively produces an *in situ* "snap-shot" of the molecular position; therefore, it may represent a range of tilts of different direction and magnitude within and between gap regions and fibrils. These molecular configurations are likely to be influenced by the cooling rate.

Overall, the ambient samples have a higher relative intensity than the cryo-cooled samples, but the profiles of the diffuse scatter and the Bragg peaks

both indicate that the biggest change lies in the low angle (near equatorial) region (Figure 3). Electron density contrasts between the four segments in the gap region are known to contribute significantly to the diffraction intensities in this region (Fraser *et al*., 1987; Hulmes *et al*., 1995). Thus, it is shown here that cryo-cooling probably reduces or restricts the thermal lateral and rotational mobility of molecular segments particularly in the gap region, where there is a lower molecular packing density and a reduced frequency of stabilising amino acid triplet sequences (Fraser *et al*., 1983). There may also be an effect on the mobility of the telopeptides, which are also thought to contribute significantly to the low angle intensities (Fraser *et al*., 1987). Any diffuse scatter remaining after cryo-cooling may be largely attributed to static (positional) molecular disorder within the unit cells.

(b) Cryo-TEM

To investigate the presence of sub-fibrillar structures, and to monitor the effect of freezing on sample preservation, fibrillar preparations of rat tail tendon collagen were analysed by cryo-TEM. Unlike most TEM preparations of stained collagen fibrils, the banding profile in the cryo-TEM images (Figure 4) is not a product of dye interactions. Rather, it is due to the inherent electron density distribution along the fibrils: the electron-dense overlap region appearing dark and the electron-lucent gap region appearing pale. Cryo-TEM thus affords a closer approximation to the near native structure of fibrils (providing the freezing process is rapid enough to prevent ice formation and thus structural damage). The average electron density profile from seven gap-overlap repeats of the TEM cryo-section is shown in Figure 5. The N and C-termini peaks are clearly visible at either end of the overlap region on the left of the profile, with the less intense gap region on the right. There is only moderate agreement between the Fourier analyses of the cryo-TEM sections and those of simulated (model) data for orders one to six, and poor agreement for the higher orders (Figures 6 and 7). This is important as the cryo-TEM images are from unstained samples.

Acknowledgements

Dr. M. McDonald and Dr. Rob Kehoe at station 14.1, Daresbury Laboratories, UK

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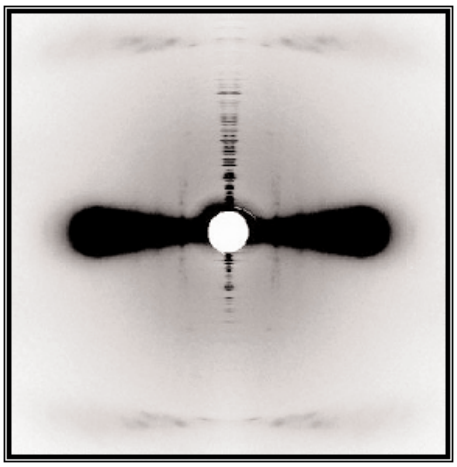
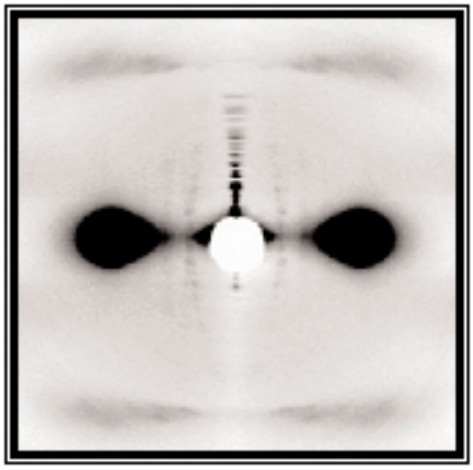
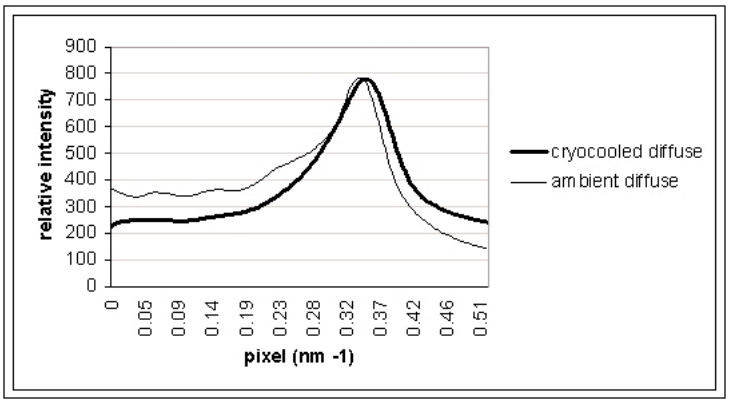
Ambient **Cryo-cooled**





Figures 1 & 2: X-ray diffraction patterns of whole rat tail tendon at (Fig 1: left) ambient (294K; 21°C) and (Fig. 2: right) near liquid nitrogen (97.5K; -176°C) temperatures. Coherent X-ray scatter in the meridional plane arises from the characteristic 67nm (D-periodic) gap-overlap step function of collagen molecules packed axially according to the quarter-stagger model (Hodge and Petruska, 1963). Bragg peaks overlaid with diffuse scatter are observed along the equator and these arise from lateral molecular packing interactions. The resolution of the diffraction data extends to approximately 1.0 nm along the equator and 0.54 nm parallel to the meridian. Note the different equatorial profiles of diffuse scatter at the different temperatures



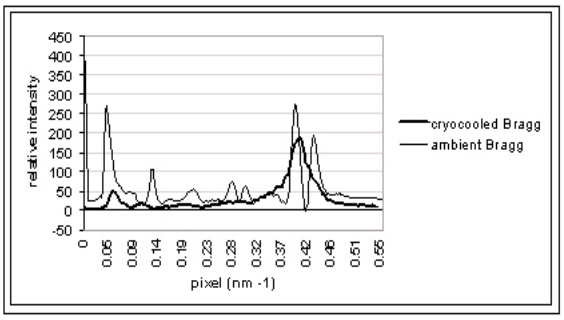


Figure 3: Equatorial profiles of diffuse (left) and Bragg (right) scatter from X-ray diffraction of whole tendon in the cryo-cooled and ambient states.

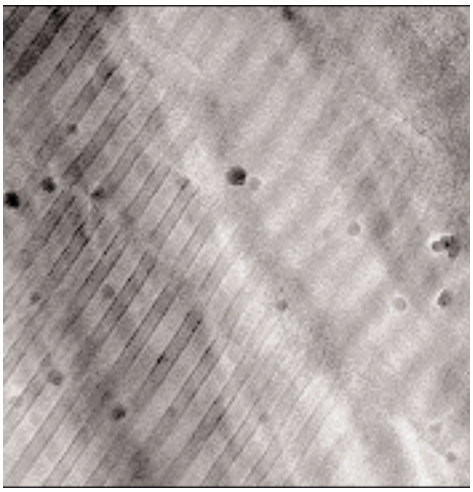


Figure 4: Transmission electron micrograph of unstained cryosectioned rat-tail tendon collagen fibrils (x 440,000). The N- and C-termini of the molecules appear as narrow (~4 nm) bands of electron dense material at either end of the overlap region. Finer (<2 nm) bands of electron dense material are also present throughout the gap region.

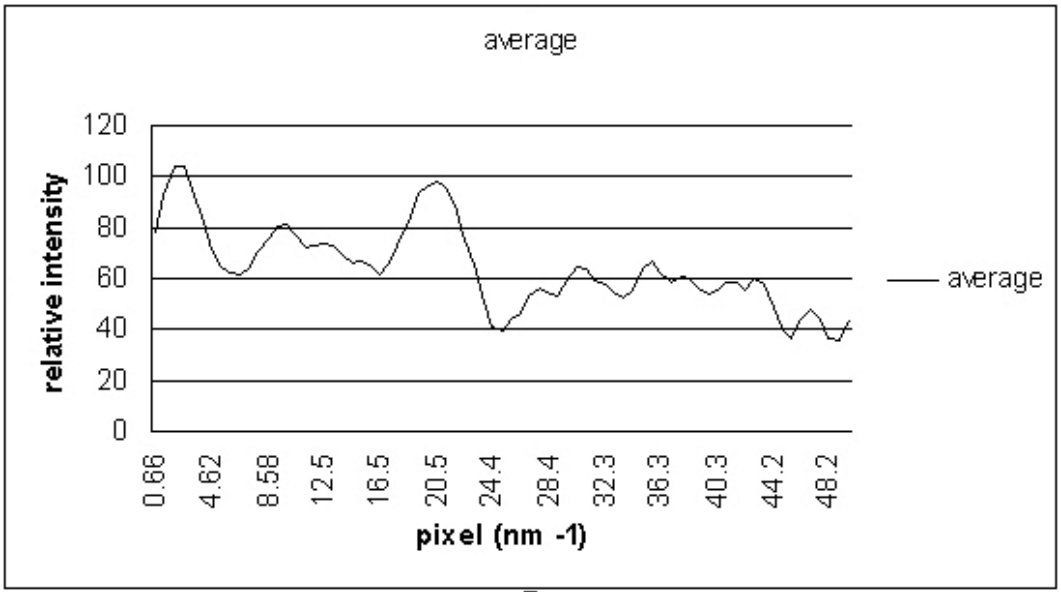


Figure 5: Average electron density profile taken from seven gap/overlap repeats shown in Figure 4.

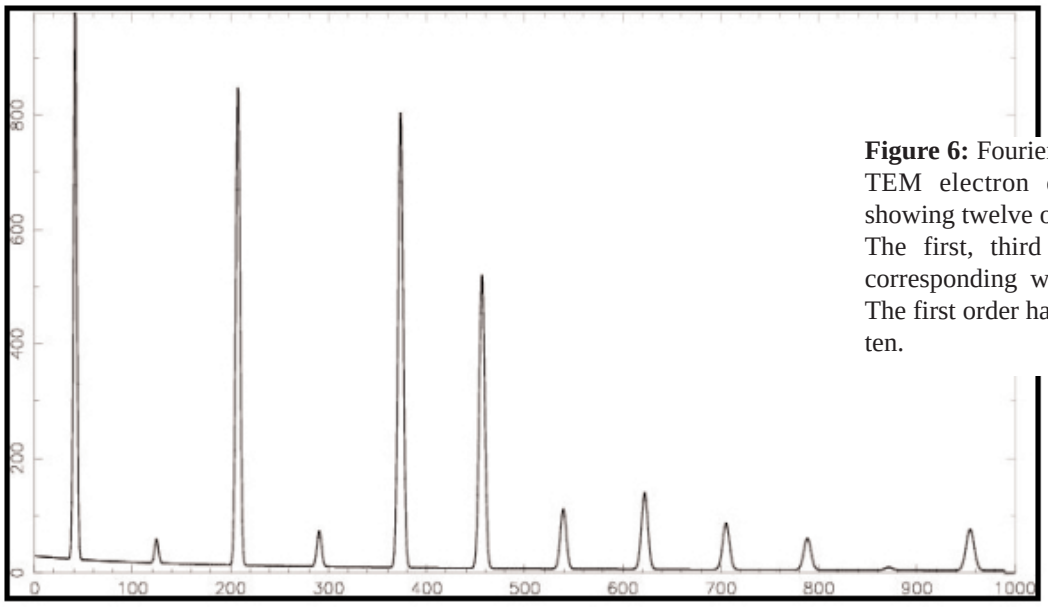


Figure 6: Fourier analysis of the averaged cryo-TEM electron density profile in Figure 5, showing twelve orders of intensity (left to right). The first, third and fifth orders are strong, corresponding with simulated data (Figure 7). The first order has been attenuated by a factor of ten.

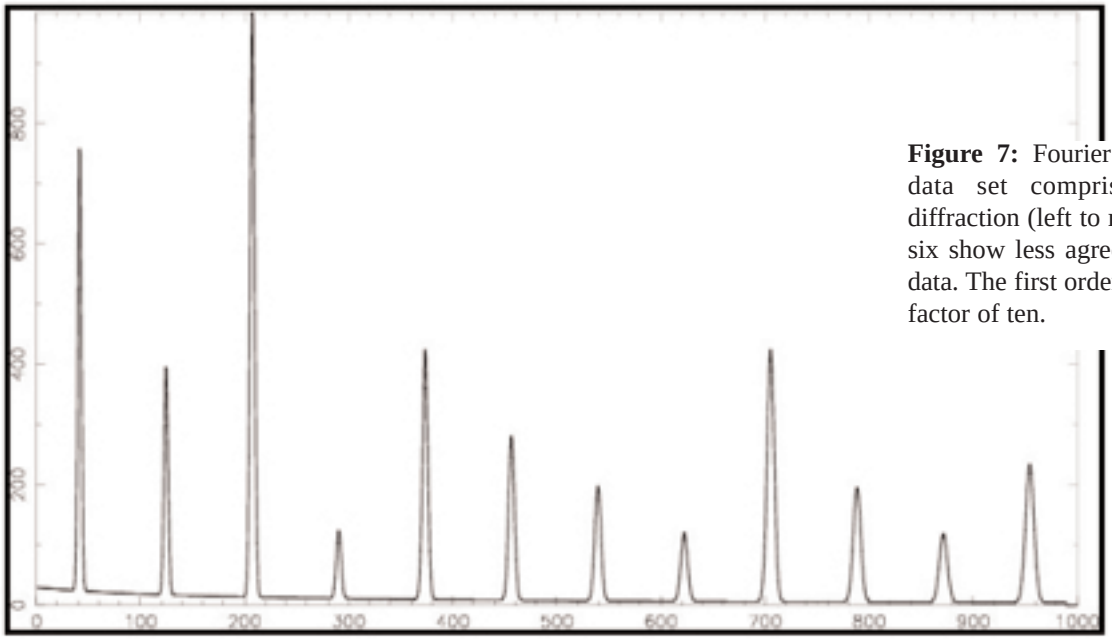


Figure 7: Fourier analysis of a simulated data set comprising twelve orders of diffraction (left to right). Orders greater than six show less agreement with the cryoTEM data. The first order has been attenuated by a factor of ten.