Osteogenesis imperfecta: an x ray fibre diffraction study

J P BRADSHAW AND A MILLER

From the Laboratory of Molecular Biophysics, Department of Zoology, Rex Richards Building, South Parks Road, Oxford; and the Department of Biochemistry, Edinburgh University Medical School, Hugh Robson Building, George Square, Edinburgh

SUMMARY The use of x ray fibre diffraction to study the molecular architecture of healthy and diseased human tendon is described. The three dimensional structure of human (finger) tendon is derived to high resolution and is shown to be very similar to that reported for rat tail tendon. In particular the presence of the 38 Å row line in the diffraction pattern suggests that a high degree of lateral order within the collagen fibrils is a more widespread feature of tendon tissue than was formerly realised. Axially projected electron density maps of the 670 Å unit repeat of the collagen fibrils of this tissue, and of tendon tissue from three cases of osteogenesis imperfecta (OI), are calculated and compared. The results are in agreement with recent biochemical studies in suggesting that type I (Sillence) OI is principally a quantitative, rather than a qualitative, defect of type I collagen biosynthesis. The features by which a molecular lesion may be recognised and characterised from diffraction data are discussed.

Key words: brittle bone syndrome, collagen, synchrotron.

Collagen is the most abundant protein of the human body. The collagenous connective tissues are responsible for preserving the structural integrity of the body and maintaining the relative positions of its component organs. It is a major constituent of tendon, ligament, blood vessels, fascia, basement and glomerular membrane, and cornea. One unfortunate consequence of this widespread distribution of the collagens throughout the body tissues is that inherited or acquired abnormalities of these proteins produce a diverse group of diseases affecting different combinations of the connective tissues. To a certain extent the pattern of each disorder reflects the tissue distribution of the affected collagen type. A good example of this phenomenon is osteogenesis imperfecta (OI), the subject of this study.

Despite being called the brittle bone syndrome OI is not necessarily restricted in its effects to bone tissue but tends to affect all of the type I collagen containing tissues, to a greater or lesser extent. This fact, together with a substantial body of previous

work, has led to the belief that the widespread disruption to the connective tissue systems of the body caused by OI is primarily caused by an abnormality in the biosynthesis of type I collagen.^{1 2}

Over the past few years it has become clear that OI is not a single disease but rather a heterogeneous collection of related conditions. Sillence has classified the syndrome into four main groups according to the prevalent features and characteristics of inheritance of each type.^{3 4} This paper reports an x ray diffraction study of the molecular basis of the condition as it occurs in tissue samples obtained from three cases of the milder, dominantly inherited form of the syndrome. This is the first time that x ray fibre diffraction has been used successfully in this type of study.

Materials and methods

Tendon samples were obtained from three patients with type I (Sillence) osteogenesis imperfecta and from one control and were kept frozen until required. The frozen samples were dissected to produce specimens of length 15 mm and diameter 0.5-1.0 mm, with the long axis aligned with the axis

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Correspondence to Dr J P Bradshaw, Department of Biochemistry, University of Edinburgh Medical School, Hugh Robson Building, George Square, Edinburgh EH8 9XD.

of the tendon. These specimens were allowed to thaw in physiological Ringer's solution before being mounted over 0.15 M NaCl in sealed specimen cells. They were stretched to remove the crimp.

Two Huxley-Holmes focused beam, x ray cameras were used, one with a specimen to film length of 175 mm, mounted on an Elliot GX13 rotating anode x ray generator, and the other of length 2000 mm, using synchrotron radiation.⁵ The use of two focal lengths allowed over 40 orders of the meridional diffraction maxima to be measured from some of the specimens, and the equatorial and off-equatorial intensity.

Each exposure was recorded on a pack of three films to ensure that the complete range of intensities could be measured. The films were processed, and their intensity data were digitised by scanning on a

Joyce Loebel Scandig 3 microdensitometer with a raster setting of 100 µm for the long camera films and 50 µm for those exposed in the shorter camera. The digitised data were corrected for background scatter and geometric distortions inherent in the optical characteristics of the measuring process, the three films from each pack were scaled to each other, and the total intensity of each reflection was integrated. The integrated intensities of corresponding reflections as measured on the two different cameras were used to scale the two sets of data.

Reciprocal lattice spacings were measured directly off the film with a Nikon profile projector travelling microscope.

Results

The corrected intensities of the meridional diffraction peaks recorded from diseased and control tendon samples are listed in Table 1. All of the data sets have been scaled to a first order intensity value of 1000. The justification for this has been given elsewhere.⁶

CONTROLS

The x ray diffraction patterns produced from human tendon (Fig. 1) were very similar to those obtained from the much studied rat tail tendon⁷ in a number of important respects. The low angle, meridional region (corresponding to the axial electron density of the diffracting structure) showed the same 670 Å repeat distance, and the relative

Table 1 Intensities of the meridional x ray diffraction peaks of tendon collagen from a variety of sources*

Order	RTT*	Control	O11	O12	O13
1	1000	1000	1000	1000	1000
2	27	23	20	11	19
3	109	98	82	106	91
4	11	12	11	6	11
5	54	55	61	63	56
6	17	17	12	17	21
7	21	31	37	37	45
8	6	16	19	19	19
9	71	66	105	70	72
10	19	14	20	20	17
11	20	8	12	11	12
12	31	29	29	22	26
13	2	2	1	3	1
14	4	7	6	7	7
15	4	8	10	11	9
16	5	11	12	13	13
17	11	6	5	8	4
18	4	5	3	2	6
19	6	3	3	3	3
20	34	35	33	30	36
21	26	28	27	28	35
22	11	8	7	13	10
23	0	7	3	6	5
24	0	3	1	3	1
25	14	19	13	13	16
26	5	6	8	5	6
27	11	8	12	10	9
28	1	4	3	3	2
29	9	8	5	7	4
30	16	13	14	11	10
31	1	5	5	7	3
32	2	4	3	6	4
33	2	4	5	9	4
34	17	12	—	—	7
35	4	5	—	—	2
36	6	5	—	—	3
37	4	9	—	—	5
38	3	6	—	—	—

*Rat tail tendon; after Hulmes *et al.*⁶

Fig. 1a

Fig. 1b

Fig. 1 Diffraction patterns of healthy human tendon, taken at specimen to film distances of (a) 175 mm and (b) 2000 mm. The 38 Å row line is indicated.

intensities of the first few diffraction peaks were comparable in the two tissues, indicating that at low resolution the diffracting structures contain the same step function of electron density. The equatorial region of the diffraction patterns (which contains the lateral packing information) was also extremely similar to the corresponding region of the

rat tail tendon pattern. Some of the control samples even produced the 38 Å row line, previously reported for only a few of the more crystalline tendon types.⁸ This suggests that the quasi-hexagonal arrangement of collagen molecules proposed by Fraser *et al*⁷ may be more widespread in terms of tendon types than was previously thought.

Comparison of the individual meridional intensities with those recorded for rat tail tendon⁶ showed that the two diffraction patterns, and therefore the two structures producing the diffraction, were extremely similar to quite a high resolution. Unfortunately it was not possible to measure the phase angles corresponding to these meridional intensities but, since the two types of tendon appear to be remarkably similar in terms of their meridional diffraction amplitudes, it is reasonable to assume that the associated phase angles would not show any marked differences between the two tissues. The phases of the first 41 meridional reflections of rat tail tendon have been determined by modelling the axial electron density of one 'D' repeat of the collagen fibril.^{6,8} Clearly these phases could also be combined with the human tendon intensity data in a Fourier synthesis to produce an axial electron density model for this type of tendon. The validity of such a procedure is dependent upon the degree of similarity of the two structures. The evidence, as outlined above, indicates that in most respects the two structures are indeed very similar. The calculated Fourier synthesis of the two tissues are shown in Fig. 2a.

The scarcity of suitable tissue meant that only one control sample was used in this study. The justification for using just one control sample for this comparative study may be found in the work of Brodsky and Eikenberry, who showed in a comparative x ray diffraction study of type I collagens from a variety of different sources that whereas collagens from different species produce subtle, but reproducible, differences in the intensities of the meridional diffraction peaks, within any single species the degree of similarity between tendons from different anatomical locations is very high.⁹ This is borne out by the fact that type I collagen accounts for over 90% of the dry weight of tendon tissue, with the variable proteoglycans constituting only a minor fraction of the total tissue mass.¹⁰

OSTEOGENESIS IMPERFECTA

Although the three samples of OI tissue used in this study came from different patients, and despite previous observations that the syndrome is a heterogeneous group of related conditions,^{1,2} the diffraction patterns obtained from the three tissue samples were extremely similar. Moreover, all three patterns

Fig. 2a

Fig. 2b

were almost indistinguishable from that collected from the control specimen. The Fourier syntheses of the meridional diffraction, calculated as for the control samples, also showed strong resemblances to that of the control (see Figs 2b-d).

Discussion

The use of x ray fibre diffraction methods to investigate connective tissue disease is dependent upon one fundamental assumption: that changes in the biosynthesis of collagen, of the type brought

about by the diseases studied, will show their presence by altering the diffraction patterns obtained from samples of the diseased tissues. This poses two important questions. Firstly, in what ways might such a biosynthetic defect affect the diffraction pattern of collagen? Related to this is the second question: how small a change could the technique be expected to detect?

The well defined, axial, one dimensional crystallinity of the collagen fibril, with its repeat distance of 670 Å, produces an equally well defined reciprocal lattice of non-zero points, spaced at intervals of 670

Fig. 2c

Fig. 2d

Fig. 2 Fourier syntheses of the axially projected electron density of collagen fibrils from a variety of sources. The ordinate represents the electron density of each collagen molecule per unit axial translation.

$\text{\AA}^{-1}$ reciprocal lattice units along the meridian of the diffraction pattern. The width of each reciprocal lattice point in any particular direction is inversely related to the number of consecutive unit cells in that direction which are contributing to the diffraction peak. This number is very large in the axial direction, and the reflections are therefore well defined and quite narrow. In the equatorial plane, however, the number is relatively small since

collagen fibrils only measure 1–2000 $\text{\AA}$ in diameter, and it is unlikely that the crystallinity is preserved across the whole width.¹¹ Each meridional reflection may therefore be considered to be disc shaped. The tendon diffraction pattern is the collective product of a large number of individual fibres, and the lateral spread of the meridional peaks may be exaggerated if the diffracting fibrils do not all share the same axis. The effect of this fibrillar disorienta-

tion is to spread the meridional intensities out into arcs centred on the origin of the reciprocal lattice. This provides the first diagnostic feature of the tendon diffraction pattern, for the size of the meridional peaks and their degree of arcing disclose both the underlying extent of crystalline order and the degree of fibrillar disorientation. Thus modifications to the connective tissue components which disrupt the molecular architecture of the fibril will betray their presence in the diffraction patterns produced by the affected tissues. Disruptions of a larger scale will be shown by an increase in the level of general background blackening of the film. The resulting diffraction pattern would have a very poor signal to noise ratio.

If, while maintaining a high degree of structure in the collagen fibril, a connective tissue disease manifests itself by modifying the nature of that structure then a corresponding modification will be shown in the diffraction pattern. Changes in the lateral packing of molecules, or in their 'D' stagger, will affect the equatorial or meridional spacings respectively, while alterations in the peptide chain conformation or composition will become evident from the intensities of the meridional peaks. As shown by Hulmes and his coworkers the intensities of the first few meridionals are dominated by the relative lengths of the gap and overlap regions of the collagen fibril.⁶ Changes in these intensities show altered proportions of the two regions. Similarly, the non-helical telopeptide extensions are known to have their prevailing influence on orders in the region between 8 and 19. The higher orders of diffraction contain structural information of a higher resolution. Localised perturbations of the molecular structure would be expected to affect these intensities in a systematic fashion: an abnormality of the n th order should also be evident in changes to orders $2n$, $3n$, $4n$, etc.

The second question that needs to be addressed concerns the resolution of the technique: how small a structural change could x ray diffraction be expected to detect? In absolute terms the resolution of the structural information contained in a diffraction pattern is related to the maximum angle of diffracted rays recorded. In terms of axial electron density of the collagen fibril the degree of fine detail is limited by the number of meridional intensities captured on the film. In practice, however, the situation is not so simple for it is necessary to bear in mind two other factors, one a feature of connective tissue diseases, and one a feature of x ray diffraction. In using x ray diffraction to study connective tissue disease we are looking for changes at a molecular level which, when magnified by the interaction of the defective component with other

similarly affected molecules, are capable of causing the widespread disruption so characteristic of connective tissue disorders. Thus even if the technique is not able to detect subtle electron density changes brought about by a single amino acid substitution it certainly would be capable of detecting any larger scale disruptions to the molecule or fibril brought about by the substitution. Furthermore, it is an inherent feature of x ray diffraction that each part of the diffracting structure contributes information to every part of the diffraction pattern. In other words, even though a structural alteration may be so small that its primary contribution to the diffraction pattern occurs outside the maximum angle of data collection, it might be expected to betray its presence by its effect upon the rest of the diffraction data.

In summary, any significant structural alteration to the molecular components of tendon will affect the x ray diffraction pattern of the tissue in a predictable fashion. Of the three samples of OI tissue examined, none showed any systematic modification to the control diffraction patterns. It is therefore reasonable to assume that the structure of the type I collagen, which accounts for over 90% of their dry weight, is not significantly altered by the disease. These findings may be explained by one or more of three options: (a) The type I collagen synthesised by the tendon fibroblasts is perfectly normal. (b) The collagen produced by the cells is defective in some minor way, but the defect is too small to be detected by x ray diffraction. (c) The collagen is a heterogeneous mixture of normal and defective molecules. The good molecules are assembled into fibrils, while the bad ones are discarded at some stage of the biosynthetic process.

Let us consider these options in turn. The first one would seem to go against the majority of the previous work on the brittle bone syndrome.^{1,2} The second option may be subdivided into two possibilities. If the basis of type I OI consists of an alteration in the primary structure of collagen molecules which brings about a larger scale disruption of the structure of the fibril or tissue then this explanation would not seem to apply to the tissues examined. As was explained above the primary alteration might be invisible to the techniques employed but the ensuing disruption would not. This option does also cover another possibility. The modification to the primary structure might bring about its effect not by disrupting the fibril structure but by delaying the production of fibrils. The disease would then manifest itself by a quantitative rather than a qualitative defect of type I collagen fibrils. Such a defect would not be detectable by present x ray diffraction methods for the fibril structure examined

would appear quite normal. A similar effect would ensue from the third option, in which the primary defect is sufficiently substantial to prevent some of the collagen precursors from completing their biosynthetic pathway.

Our data therefore indicate that in the samples studied the effects of type I OI are best understood in terms of a decrease in the total amount of normal type I collagen fibrils within the connective tissues, rather than a structural defect in those fibrils. These findings are in agreement with previous work on this milder form of the brittle bone syndrome. Assays of the different collagens in skin tissue and fibroblast culture have shown that the ratio of type III collagen to type I is increased.¹²⁻¹⁴ It has been suggested that this might be brought about by a non-functional allele for the pro- $\alpha 1(I)$ chain.¹² Normal type I collagen is produced, but at approximately half the normal rate. The excess $\alpha 2(I)$ chains appear to be degraded intercellularly, there being no evidence for the formation of hybrids incorporating these chains, such as $\alpha 1(I).$ $\alpha 2(I)_2$.¹⁵ This is essentially the same scheme as that first proposed by Sykes *et al.*¹⁶

This study serves to illustrate the great potential offered by *x* ray fibre diffraction for the investigation of connective tissue disorders. When compared with electron microscopy the method has higher resolution and the sample is maintained in a state which closely approximates physiological conditions. The data produced contain structural information at both the resolution of the primary molecular lesion and on the larger scale at which the disruption is manifest. The forte of *x* ray diffraction is the detection of order, and as such it is an ideal tool for the characterisation of disruptions to the molecular architecture of connective tissues by disease.

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References

- 1 Smith R, Francis M J O, Houghton G R. *The brittle bone syndrome*. London: Butterworth, 1983.
- 2 Prockop D J, Kivirikko K I. Heritable diseases of collagen. *N Engl J Med* 1984; **311**: 377-86.
- 3 Sillence D O, Senn A, Danks D M. Genetic heterogeneity in osteogenesis imperfecta. *J Med Genet* 1979; **16**: 101-16.
- 4 Sillence D O. Osteogenesis imperfecta: an expanding panorama of variants. *Clin Orthop* 1981; **159**: 11-25.
- 5 Nave C, Helliwell J R, Moore P R, *et al.* Facilities for solution scattering and fibre diffraction at the Daresbury S.R.S. *J Appl Cryst* 1985; **18**: 396-403.
- 6 Hulmes D J S, Miller A, White S W, Brodsky Doyle B. Interpretation of the meridional *x* ray diffraction pattern from collagen fibres in terms of the known amino-acid sequence. *J Mol Biol* 1977; **110**: 643-66.
- 7 Fraser R D B, Macrae T P, Miller A, Suzuki E. Molecular conformation and packing in collagen fibres. *J Mol Biol* 1983; **167**: 497-521.
- 8 Hulmes D J S, Miller A, White S W, Timmins P A, Berthet-Colominas C. Interpretation of the low angle meridional neutron diffraction patterns from collagen fibres in terms of the amino-acid sequence. *Int J Biol Macromol* 1980; **2**: 338-46.
- 9 Brodsky B, Eikenberry E F. Characterisation of fibrous forms of collagen. *Methods Enzymol* 1982; **52**: 127-74.
- 10 Mathews M B. *Connective tissue. Molecular biology, biochemistry and biophysics*. Berlin: Springer, 1975: 19.
- 11 Miller A, Wray J S. Molecular packing in collagen. *Nature* 1971; **230**: 437-9.
- 12 Barsh G S, David K E, Byers P H. Type I osteogenesis imperfecta: a non-functional allele for proalpha 1(I) chains of type I procollagen. *Proc Natl Acad Sci USA* 1982; **79**: 3838-42.
- 13 Pope F M, Nicholls A C. *Mainz symposium on bone dysplasias*. 1982. (Abstract).
- 14 Rowe D W, Poirier M, Shapiro J R. Osteogenesis imperfecta: a genetic probe to study type I collagen biosynthesis. In: Veis A, ed. *Chemistry and biochemistry of mineralised connective tissues*. Amsterdam: Elsevier, 1981: 155-62.
- 15 Barsh G S, Byers P H. Reduced secretion of structurally abnormal type I procollagen in a form of osteogenesis imperfecta. *Proc Natl Acad Sci USA* 1981; **78**: 5142-6.
- 16 Sykes B C, Francis M J O, Smith R. Altered relation of two collagen types in osteogenesis imperfecta. *N Engl J Med* 1977; **296**: 1200-3.