Collagen fibrils *in vitro* grow from pointed tips in the C- to N-terminal direction

Karl E. KADLER,* Yoshio HOJIMA and Darwin J. PROCKOP

Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Philadelphia, PA 19107, U.S.A.

Growth of collagen fibrils was examined in a system in which collagen monomers are generated by specific enzymic cleavage of type I pCcollagen with procollagen C-proteinase. Fibrils formed at 37 °C had highly tapered and symmetrical pointed tips. The pattern of cross-striations in the pointed tips indicated that all the molecules were oriented so that the N-termini were directed towards the tip. At 29 °C and 32 °C, the fibrils formed were thicker. One end of fibrils formed at 29 °C was blunt, and the other was pointed. Growth of the fibrils was exclusively from pointed tips. Occasionally a spear-like projection appeared at a blunted end. The spear-like projection then became a new pointed tip for growth in the opposite direction. The results suggested a model for fibril growth with at least three distinct binding sites for monomers. In the model, the pointed tip is the site with the highest affinity for the binding of monomers and most probably defines the critical concentration for fibril assembly. The main shaft of the fibril is a site with very low affinity for binding. The blunted end defines a low-affinity binding site where monomers can bind in opposite orientation to produce growth from a new pointed end.

INTRODUCTION

The ability to generate large structures by the self-assembly of protein monomers is a fundamental feature of most living organisms. Among the largest and most abundant of such structures are the fibrils of type I collagen found in skin, tendons, bone and other connective tissues of vertebrates [1]. The self-assembly of type I collagen into fibrils [2,3] differs from other protein self-assembly systems, such as assembly of tobacco mosaic virus and actin filaments [4], in that the final polymeric structure of collagen is formed by addition of a rod-like monomer in both a longitudinal and a lateral direction. In spite of extensive analyses, it is still uncertain how the fibrils grow [1–3,5–13]. Moreover, growth of individual collagen fibrils has never been observed.

We have recently developed a new system [3,14,15] for studying assembly of fibrils of type I collagen in which the collagen monomer is generated *de novo* by specific enzymic cleavage of the partially processed precursor of collagen called pCcollagen. The system can be used to generate the collagen monomer in solution in a highly reproducible manner, and the assembly of fibrils can be examined in a physiological buffer at temperatures in the range 26–41 °C. Here we have examined the morphology of the fibrils formed with negative-staining electron microscopy and the growth of fibrils by dark-field light microscopy. The results demonstrate that all type I collagen fibrils initially formed *in vitro* have blunted and pointed ends, and that they grow only from their highly tapered and symmetrical pointed ends.

EXPERIMENTAL

Preparation of procollagen

¹⁴C-labelled type I procollagen was purified from the medium of cultured fibroblasts from normal human skin as described previously [14]. In brief, confluent cultures were incubated in Dulbecco's modified Eagle's medium containing 1 μ Ci of a U-

¹⁴C-labelled mixture of L-amino acids/ml, 25 μ g of L-ascorbic acid/ml and no fetal-bovine serum. Proteins were precipitated from the medium with (NH₄)₂SO₄, and the type I procollagen was purified by chromatography on two successive columns of DEAE-cellulose [16,17]. Purified procollagen was concentrated by pressure ultrafiltration and stored at –15 °C in 0.4 M-NaCl/0.04 % NaN₃/0.1 M-Tris/HCl buffer, pH 7.4 at 20 °C. Procollagen concentration was determined by colorimetric assay [18,19], assuming 10.11 % hydroxyproline by weight for type I procollagen [17].

Preparation of pCcollagen

Type I pCcollagen was prepared by digesting 1 mg of purified type I procollagen with partially purified procollagen N-proteinase [20] and isolating the pCcollagen by gel filtration [3]. In some experiments in which the type I procollagen was not completely cleaved by the N-proteinase, the pCcollagen was rechromatographed at 4 °C on a column of DEAE-cellulose (1 cm $\times$ 1.6 cm diameter) equilibrated with 2 M-urea and 0.01 % NaN₃ in 0.075 M-Tris/HCl buffer, pH 7.8 at 20 °C.

C-proteinase

The enzyme was purified as described previously [21] and had 300 units of activity/ml in the fibril formation buffer. One unit was defined as the amount of enzyme that cleaves 1 μ g of type I procollagen at 35 °C in a reaction system with a substrate concentration of 12 μ g/ml [21]. In experiments performed at different temperatures, the concentration of C-proteinase was adjusted to keep constant the rate of cleavage of the pCcollagen substrate [14].

N-proteinase

The enzyme was partially purified from the leg tendons of 400 dozen 17-day chick embryos as described previously [20]. The enzyme had 1700 units of activity/ml.

*Present address: Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, U.K.

Fibril formation

The method of initiating fibril formation was the same as described previously [3]. In brief, pCcollagen substrate and C-proteinase were dialysed against 600 vol. of fibril-formation buffer, which was a modified Dulbecco's modified Eagle's medium and consisted of 20 mM- NaHCO_3 , 117 mM- NaCl , 5.4 mM- KCl , 1.8 mM- CaCl_2 , 0.81 mM- MgSO_4 , 1.03 mM- NaH_2PO_4 and 0.01 % NaN_3 , pH 7.3 at 20 °C. To initiate fibril formation, the pCcollagen and C-proteinase solutions were mixed in a micro-centrifuge tube at 4 °C, and the tube was charged with water-saturated CO_2/air (1:9). The micro-centrifuge tube was placed at the experimental temperature for 5 min. The solution was then transferred to a preheated chamber suitable for examining the growth of fibrils by dark-field light microscopy [15]. The chamber was constructed from a 2 mm $\times$ 5 mm-diameter plastic cylinder affixed to a microscope slide by epoxy glue. The plastic cylinder was obtained by cutting off the top 2 mm of a

200 μl pipette tip with a razor blade. The top of the chamber was sealed with a glass coverslip and grease.

The microscope slide with chamber was placed on a heating stage (Linkam) which had a 5 mm-diameter hole at its centre. The heating stage and chamber were placed on a Zeiss light-microscope stage, and the growth of the fibrils was examined by dark-field illumination. At times during an experiment, photomicrographs were taken with a $\times 6.3$ objective and a $\times 10$ eyepiece lens, and the images were recorded on Kodak Panatomic 32 ASA film. The negatives were printed, and overlapping montages extending over the whole of the chamber were constructed for each time point.

Electron microscopy

The pCcollagen substrate (50 $\mu\text{g}/\text{ml}$) and the C-proteinase (10 units/ml) were mixed in a micro-centrifuge tube, and the tube was charged with water-saturated CO_2/air (1:9). The

Fig. 1. Electron micrographs of collagen fibrils formed

The fibrils were formed by incubating 50 μg of pCcollagen/ml with 10 units of procollagen C-proteinase/ml at 37 °C for 6 h. The fibrils were partially fixed by adding 0.1 vol. of 37 % formaldehyde and 0.1 vol. of 300 mM-sodium phosphate/100 mM-sodium acetate (pH 7.0). The samples were incubated at room temperature for 30 min. The samples were then transferred to carbon-coated grids and stained with 2 % sodium phosphotungstate (pH 7.0) for 30 s. Bar represents 4.4 μm or 300 nm.

reaction mixture was incubated at 37 °C for 6 h. Then 0.1 vol. of 37% (v/v) formaldehyde and 0.1 vol. of 300 mM- NaH_2PO_4 /100 mM-sodium acetate/0.01% NaN_3 (pH 7.0 at 20 °C) was added, and the mixture left at room temperature for 30 min. A drop of the solution was placed on a carbon-coated copper grid and left to adsorb for 5 min. The grid was blotted and the sample negatively stained with 2% (w/v) sodium phosphotungstate, pH 7.0. The sample was examined with a Zeiss model EM109 electron microscope operated at an accelerating voltage of 80 kV and at a magnification of $\times 3000$ –50 000. Images were recorded on Kodak TP120 film.

RESULTS

Electron microscopy of fibrils

The fibrils formed at 37 °C were thin, round and flexible, and they were indistinguishable from type I collagen fibrils seen in tissues [15]. When examined with either dark-field or difference interference contrast (Nomarski) light microscopy, many of the fibrils appeared to have pointed tips [15]. Here we used negative-staining electron microscopy to define the ends of the fibrils.

The main shafts of the fibrils had uniform diameters and a sharply defined cross-striated pattern characteristic of fibrillar collagens (bottom panel in Fig. 1). Most of the ends of fibrils were highly tapered and symmetrical (top panels in Fig. 1). The symmetrical appearance and sharp cross-striated pattern persisted up to the point where the tapering ends were no longer

observed. The smallest tips seen were about one-third of a D-unit, and therefore about 25 nm wide, or about 15 times the diameter of a monomer of type I collagen [1]. In all the pointed tips examined, the pattern of cross-striations indicated that all the molecules were oriented so that the *N*-termini were directed toward the tip. A few of the ends of the fibrils had irregular and blunted ends (results not shown).

Light-microscopy: growth of individual fibrils

To examine growth of individual fibrils, fibrils were formed in small chambers [15] suitable for light-microscopy with dark-field optics. Initial experiments carried out at 37 °C suggested that the first fibrils detected grew longitudinally from pointed ends that were similar to the pointed ends seen by negative-staining electron microscopy. The fibrils formed at 37 °C, however, were too thin to distinguish the ends clearly. Therefore, subsequent experiments were performed at 29 °C or 32 °C, temperatures at which the fibrils formed were thicker [15]. In the first series of experiments, fibrils were generated at 29 °C and the system was allowed to come to equilibrium (Fig. 2). All the fibrils had pointed and blunted ends. Also, all had about the same maximal diameters as observed by dark-field or Nomarski optics (results not shown). Some of the fibrils appeared to aggregate through their blunt ends to form V-shaped or star-like structures.

As they first appeared in the sample, the fibrils had readily discernible blunted and pointed ends (Fig. 3). Also, they rapidly reached a maximal diameter. The fibrils grew exclusively from

Fig. 2. Dark-field light microscopy of type I collagen fibrils formed at 29 °C

The fibrils were generated by incubating 50 μg of pCcollagen/ml with 26 units of procollagen C-proteinase/ml. The system was allowed to come to equilibrium for 22 h. The photograph presents all the fibrils in the sample.

Fig. 3. Growth of fibrils at 29 °C under conditions as in Fig. 2

Growth of four fibrils was observed at 4 h (a), 5 h (b), 6 h (c), 10 h (d) and 24 h (e). Small air bubbles help orient the fibrils. Note growth at pointed ends exclusively.

Fig. 4. Asymmetric growth of collagen fibrils at 32 °C

Fibrils were generated by incubating 50 μ g of pCcollagen/ml with 18 units of C-proteinase/ml. At 4 h (a) a single fibril with a blunted and a pointed end is shown. At 5 h (b) a spear-like projection is seen at the blunted end of the fibril. At 6 h (c), at 10 h (d) and at 24 h (e) growth from both ends of the fibril is seen.

the pointed ends and without any further increase in the maximal diameter. Occasionally, a spear-like projection appeared at a blunted end (Fig. 4). Then the spear-like projection became a pointed end for growth in the opposite direction. The number of fibrils that developed spear-like projections on blunt ends and then grew in both directions was larger when higher concentrations of pCcollagen substrate were used (results not shown) and when the fibrils were formed at higher temperatures (Fig. 4).

The tips of the fibrils grew more slowly as the temperature

decreased (compare tip growth at 29 °C in Fig. 3 and at 32 °C in Fig. 4), but it was not apparent whether the decreased rate of growth at the tips was related to the increase in diameters of the fibrils.

DISCUSSION

The results here are consistent with recent observations by Birk *et al.* [13], who defined the ends of individual type I collagen

fibrils by analysing electron micrographs of serial sections of leg tendons from chick embryos. Both ends of individual fibrils were pointed, but the fibrils were asymmetric in that the maximal diameter of a fibril was closer to one end than the other. Therefore the data were consistent with the observations here that fibrils grow unidirectionally from pointed tips, but that bidirectional growth can occur if monomers in the reverse orientation bind to a blunted end, and then form the equivalent of a new pointed tip for growth in the opposite direction (Fig. 4).

The results suggest a model of the fibril with a minimum of three distinct sites for the addition of monomers. The pointed tip of the fibril is the site with the highest affinity for binding of monomers, and probably defines the critical concentration for fibril assembly, previously shown to be 1 nM (0.5 µg/ml) at 37 °C [3]. The main shaft of the fibril does not grow in diameter after initial growth of the fibril, and therefore is likely to have a very low affinity for binding of monomers. The blunt end of the fibril is likely to be a site of intermediate affinity to which monomers can bind in the reverse orientation. After addition of a monomer in the reverse orientation, the blunt end becomes equivalent to a pointed tip for growth in the opposite direction to the initial growth.

Longitudinal growth of type I collagen fibrils is largely directed by repeated clusters of hydrophobic and hydrophilic residues that divide the monomer into 4.4 D-units and cause monomers to associate so that each is staggered by one D-unit or a multiple of D-units relative to its nearest neighbours [1]. Lateral growth, however, is poorly understood. Currently there are two views about the lateral packing of collagen fibrils [1,9–12]. One view is that the packing is ordered and defined by a regular filamentous substructure of microfibrils. The alternative view is that the lateral packing occurs without an intermediate structure, that disorder is common, and that crystalline domains are formed only in selective conditions. Since the results here demonstrated that fibrils grow from highly tapered and symmetrical tips, they strongly support the suggestion that lateral packing is highly ordered. Therefore, it is very likely that lateral packing is directed by an intermediate structure. The intermediate structure apparently controls systematic addition of monomers to the pointed tips, but sharply limits the maximal diameter of the fibrils that can be formed under a given set of conditions.

The growth of collagen fibrils from pointed tips suggests that the molecular principles are similar to those involved in generating many other large structures in Nature such as snow flakes

and the viscous fingers seen in fluid mechanics that grow from pointed tips and frequently generate fractals [22,23].

This work was supported in part by N.I.H. research grant AR-38188.

REFERENCES

1. Piez, K. A. (1984) in *Extracellular Matrix Biochemistry* (Piez, K. A. & Reddi, A. H., eds.) pp. 1–40, Elsevier, New York
2. Cooper, A. (1970) *Biochem. J.* **118**, 355–365
3. Kadler, K. E., Hojima, Y. & Prockop, D. J. (1987) *J. Biol. Chem.* **262**, 15696–15701
4. Lauffer, M. A. (1975) in *Entropy-Driven Processes in Biology* (Kleinzell, A., Springer, G. F. & Wittmann, H. G., eds.), vol. 20, Chapman and Hall, London
5. Gross, J. & Kirk, D. (1958) *J. Biol. Chem.* **233**, 355–360
6. Comper, W. D. & Veis, A. (1977) *Biopolymers* **16**, 2113–2131
7. Gelman, R. A., Williams, B. R. & Piez, K. A. (1979) *J. Biol. Chem.* **254**, 180–186
8. Silver, F. H., Langley, K. H. & Trelstad, R. L. (1979) *Biopolymers* **18**, 2523–2535
9. Hulmes, D. J. S. & Miller, A. (1979) *Nature (London)* **282**, 878–880
10. Fraser, R. D. B. & MacRae, T. E. (1981) *Int. J. Biol. Macromol.* **3**, 193–200
11. Holmes, D. F. & Chapman, J. A. (1979) *Biochem. Biophys. Res. Commun.* **87**, 993–999
12. Hulmes, D. J. S., Holmes, D. F. & Cummings, C. J. (1985) *J. Mol. Biol.* **184**, 473–477
13. Birk, D. E., Zycband, E. I., Winkelmann, D. E. & Trelstad, R. L. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4549–4553
14. Kadler, K. E., Hojima, Y. & Prockop, D. J. (1988) *J. Biol. Chem.* **263**, 10517–10523
15. Kadler, K. E., Hulmes, D. J. S., Hojima, Y. & Prockop, D. J. (1990) *Ann. N.Y. Acad. Sci.*, in the press
16. Peltonen, L., Palotie, A. & Prockop, D. J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 6179–6183
17. Fiedler-Nagy, C., Bruckner, P., Hayashi, T. & Prockop, D. J. (1981) *Arch. Biochem. Biophys.* **212**, 668–677
18. Kivirikko, K. I., Laitinen, O. & Prockop, D. J. (1967) *Anal. Biochem.* **19**, 249–255
19. Berg, R. A. (1982) *Methods Enzymol.* **82**, 372–393
20. Hojima, Y., McKenzie, J., van der Rest, M. & Prockop, D. J. (1989) *J. Biol. Chem.* **264**, 11336–11345
21. Hojima, Y., van der Rest, M. & Prockop, D. J. (1985) *J. Biol. Chem.* **260**, 15996–16003
22. Meakin, P. (1985) in *On Growth and Form: Fractal and Non-Fractal Patterns in Physics* (Stanley, H. E. & Ostrowsky, N., eds.), Nijhoff, Dordrecht
23. Nittmann, J. & Stanley, H. E. (1986) *Nature (London)* **321**, 663–668

Received 16 October 1989/11 December 1989; accepted 4 January 1990