Procollagen type III *N*-terminal endopeptidase in fibroblast culture

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A peptidase activity capable of excising in a single fragment the *N*-terminal extension of the precursor of collagen type III (p-N-collagen type III) was observed in calf tendon fibroblast culture medium. A new procedure was developed for detecting this peptidase (p-N-collagen type III peptidase). It is based on the use of ^{14}C -labelled p-N-collagen type III obtained by carboxymethylation of the half-cystine residues with iodo- ^{14}C acetamide. The released labelled *N*-terminal extension is soluble in 27% (v/v) ethanol, whereas the uncleaved substrate and the collagen are precipitated under these conditions. The endopeptidase nature of p-N-collagen type III peptidase is supported by the similarity in molecular weight of the product of cleavage of p-N-collagen III by the enzyme to those obtained by cleavage with bacterial collagenase. An apparent K_m of $0.3 \times 10^{-6} \text{ M}$ was established. The pH optimum of p-N-collagen type III peptidase is similar to that of p-N-collagen type I peptidase, i.e. about 7.5. Both peptidases are inhibited by dithiothreitol and by Cu^{2+} and Zn^{2+} , but not by other bivalent ions. p-N-collagen type III peptidase does not cleave p-N-collagen I or p-N-gelatin I. Partial purification of p-N-collagen type III peptidase from fibroblast culture medium was performed by sieve chromatography on Ultrogel AcA-34 to yield two peaks of activity, of mol.wts. 170 000 and 100 000. Part of the activity was retained on affinity chromatography on concanavalin A–Sepharose. Studied as a function of the age of the culture, p-N-collagen type III peptidase activity produced by tendon fibroblasts parallels that of p-N-collagen type I peptidase and collagen synthesis.

Like other species of collagen, type III collagen is synthesized as a precursor extended at both extremities of its central helical part by large polypeptide moieties (Byers *et al.*, 1974; Lenaers & Lapière, 1975; Timpl *et al.*, 1975). The precursor sequences are to a large extent excised and separated from the triple helix in polymeric type III collagen, as in skin and blood vessels.

Structurally the *N*-terminal extension of type III procollagen is similar to that of type I procollagen (Engel *et al.*, 1977; Brückner *et al.*, 1978). It is formed of three domains displaying different size and physicochemical structure. The most *N*-terminal region of the extension seems to be random-coiled and contains half-cystine residues in an amount similar to that in the type I *N*-terminal extension. A segment of repeating triplets (eight to ten Gly-X-Y triplets for p-N-collagen type III) confers the second

region with a helical configuration in the native state. A short peptide terminated by the cleavage site for procollagen endopeptidase connects the extension to the telopeptide. Different amino acid compositions of the *N*-terminal extensions of procollagens type I and type III as well as different immunological properties indicate that the amino acid sequences of p-N-collagen type I and p-N-collagen type III are dissimilar. In type I procollagen, a specific endopeptidase cleaves the *N*-terminal extension at a Pro–Gln bond (Hörlein *et al.*, 1978). For type III procollagen, the peptide bond of the cleavage site has not yet been identified.

We have developed a new procedure for detecting procollagen peptidases and observed in culture medium of fibroblasts a proteolytic activity capable of excising specifically the *N*-terminal extension of procollagen type III. We report below its endopeptidase mode of action, some kinetic parameters and a preliminary purification of this enzyme, which differs from procollagen type I endopeptidase.

Abbreviations used: p-N, *N*-terminal precursor peptide extension of collagen type I or type III; SDS, sodium dodecyl sulphate.

Materials and methods

Materials

N-Ethylmaleimide, phenylmethanesulphonyl fluoride and various chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany); gel-electrophoresis chemicals were from Bio-Rad Laboratories (Richmond, CA, U.S.A.); iodo[1-¹⁴C]acetamide (10 Ci/mol) was from New England Nuclear (Boston, MA, U.S.A.); dithiothreitol was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); Ultrogel AcA-34 was from LKB Produkter (Stockholm, Sweden); concanavalin A-Sepharose was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden); powdered Dulbecco's modified Eagle's medium, sera and other tissue-culture chemicals were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.); α -methyl D-mannoside was purchased from Fluka (Buchs, Switzerland); CNBr was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.); liquid-scintillation reagent (Aqualuma) was purchased from Lumac (Vel, Belgium); bacterial collagenase (form III) was obtained from Bioadvance Manufacturers (Lynbrook, NY, U.S.A.).

Procollagen substrate

Procollagen type III (p-N-collagen type III) was extracted from ground dermosparactic calf skin (it can also be collected from foetal calf skin) by extraction in 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, containing a 'cocktail' of proteinase inhibitors (20 mM-EDTA, 0.5 mM-*N*-ethylmaleimide and 0.1 mM-phenylmethanesulphonyl fluoride). The tissue extract was adjusted to 20% saturation with (NH₄)₂SO₄ and the precipitate was collected by centrifugation (at 10000g for 30 min). It was dissolved in 0.3 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, and dialysed three times against the same buffer. After removal of the undissolved material by centrifugation, the solution was adjusted to 1.5 M-NaCl, and the resulting precipitate, containing p-N-collagen type III and collagen type III, was collected by centrifugation. After the precipitate had been washed with 3 vol. of 1.5 M-NaCl, it was dissolved in 0.5 M-NaCl, and precipitation was repeated at least once. Collagen and p-N-collagen were finally precipitated from the solution by dialysis against 10 mM-K₂HPO₄, collected by centrifugation, solubilized in 0.3 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, and dialysed against large volumes of the same buffer. The purity of the preparations was monitored by SDS/polyacrylamide-disc-gel electrophoresis by the procedure of Laemmli (1970), modified by the presence of 4 M deionized urea in the separation gel.

Typical preparations were composed of approx. 90% p-N-collagen type III α_1 -chains and 10%

collagen type III α_1 -chains when collected from dermosparactic skin and of approx. 50% p-N-collagen type III α_1 -chains and 50% collagen type III α_1 -chains when extracted from foetal calf skin. They were carboxymethylated by using iodo[¹⁴C]-acetamide as described for p-N-collagen type I (Nusgens & Lapière, 1979). The results presented below refer to dermosparactic p-N-collagen type III except when otherwise indicated. Similar results (not shown) were obtained in duplicate experiments with foetal calf skin p-N-collagen type III.

The location of the precursor sequence at the *N*-terminal side of the molecule was determined by selected proteolysis of the purified [¹⁴C]carboxymethylated substrate with chicken skin collagenase before and after treatment with pepsin under the conditions described in the legend of Fig. 1.

Procollagen endopeptidase assay

The assay for p-N-collagen type III peptidase activity was similar to that described for p-N-collagen type I peptidase (Nusgens & Lapière, 1979) except for the substrate, which is [¹⁴C]carboxymethylated p-N-collagen type III (specific radioactivity 0.7×10^6 – 1.5×10^6 c.p.m./ μ mol of hydroxyproline). The reaction mixture contained 30 μ g of [¹⁴C]carboxymethylated p-N-collagen type III and the tested solution in a total volume of 250 μ l in NaCl/CaCl₂/Tris/HCl buffer (0.15 M-NaCl/2 mM-CaCl₂/50 mM-Tris/HCl buffer, pH 7.4) containing 0.5 mM-phenylmethanesulphonyl fluoride and 2.5 mM-*N*-ethylmaleimide. After incubation at 26°C the reaction was stopped by cooling and adding to the incubation mixture 0.1 vol. of 1% SDS/0.1 M-dithiothreitol/0.3 M-EDTA. The reaction mixture was fractionated by ethanol precipitation at 27 and 70% (v/v). After centrifugation (at 9000g for 20 min), the radioactivity of each supernatant was measured by liquid-scintillation counting.

Tissue culture

Calf tendon fibroblasts were collected by digesting fragments of tissue from an 8-day-old normal calf in a 0.25% solution of trypsin. The cells were subcultured up to passage 3 and kept frozen in liquid N₂. Subcultures were multiplied and used between passages 10 and 14. They were grown in Dulbecco's modified minimal Eagle's medium supplemented with glutamine (2 μ mol/ml), sodium pyruvate (1.1 μ g/ml), ascorbic acid (100 μ g/ml) and foetal calf serum (15%, v/v). The culture medium was collected and replaced every other day from day 2 after plating up to day 10 after confluency, which was obtained within 5–6 days of culture.

Procollagen endopeptidase preparation and chromatographic purification

All the procedures described were performed at

4°C. The proteins were collected from the culture medium by $(\text{NH}_4)_2\text{SO}_4$ precipitation at 35, 50 and 70% saturation, and centrifugation at 4°C (at 7000g for 30 min). The 35%-saturation- $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved stepwise in 20%- and 15%-saturated $(\text{NH}_4)_2\text{SO}_4$ solution and finally in NaCl/CaCl₂/Tris/HCl buffer. Each fraction was dialysed against large volumes of this buffer and kept frozen at -20°C until used.

Further fractionation of the most active fraction [35–20%-saturation- $(\text{NH}_4)_2\text{SO}_4$] was achieved by gel filtration on an Ultrogel AcA-34 column (1.5 cm × 70 cm) equilibrated with NaCl/CaCl₂/Tris/HCl buffer. Fractions of volume 3 ml were collected at a flow rate of 16 ml/h. The absorption at 230 nm was recorded and enzyme activity was assayed on each fraction. Active fractions were further purified by affinity chromatography on concanavalin A-Sephrose as described by Tuderman *et al.* (1978) for p-N-collagen type I peptidase.

Preparation of p-N-collagen type III peptides

Foetal-calf skin p-N-collagen type III, purified as described above, was incubated under the following conditions: 16 h at 26°C with p-N-collagen type III peptidase fraction collected between 35 and 20% saturation with $(\text{NH}_4)_2\text{SO}_4$ in NaCl/CaCl₂/Tris/HCl buffer containing 2.5 mM-N-ethylmaleimide and 0.5 mM-phenylmethanesulphonyl fluoride, or 16 h at 26°C in the presence of bacterial collagenase [10 units (Mandl *et al.*, 1953) for 100 µg of collagen] in the same buffer. For comparison, p-N-collagen type I extracted from dermatosparactic skin was treated under the same conditions except that p-N-collagen type I peptidase was used. All samples were adjusted to 27% (v/v) ethanol and centrifuged at 9000g for 20 min. The supernatant was collected, freeze-dried, reduced and carboxymethylated with iodo[¹⁴C]acetamide as described for the preparation of the labelled substrate. Solutions containing the enzymes alone were processed similarly.

The molecular size of the labelled reaction products was estimated by polyacrylamide (12.5% acrylamide)-slab-gel electrophoresis in the presence of SDS by using the technique of Laemmli (1970) and a stacking gel containing polyacrylamide (3.5% acrylamide). Fluorography was performed by the technique of Bonner & Laskey (1974), with Kodak Royal X-O-mat film.

Results

As previously described by us (Lenaers & Lapière, 1975) and others (Byers *et al.*, 1974; Timpl *et al.*, 1975), the polypeptide extension of p-N-collagen type III containing most of the half-cystine residues is located at the N-terminal end of the

molecule. In our labelled substrate this was evidenced by radioautography of polyacrylamide-slab-gel electrophoretograms of chick-skin-collagenase digestion products before and after pepsin treatment (Fig. 1). The almost complete animal-collagenase digestion (Fig. 1b) produced three-quarter N-terminal labelled fragments of p-N-collagen type III α_1 -chains migrating at the position of collagen type III α_1 -chains, a small amount of collagen type III three-quarter α_1 -chains unlabelled and one-quarter fragment labelled. Pepsin digestion (Fig. 1c) converted p-N-collagen type III α_1 -chains into collagen type III α_1 -chains, and a significant amount of label was removed. Collagenase digestion of pepsin-treated p-N-collagen type III (Fig. 1d) converted almost completely collagen type III α_1 -chains into the unlabelled three-quarter N-terminal fragment and the one-quarter fragment of slightly smaller size

Fig. 1. N-Terminal location of the peptide extension of p-N-collagen type III

SDS / polyacrylamide (12.5% acrylamide) - gel electrophoretograms, showing staining for protein (S) and radioautography (R), of dermatosparactic procollagen type III (a) carboxymethylated with iodo[¹⁴C]acetamide, (b) incubated with chicken skin collagenase purified by the technique of Shinkai *et al.* (1977) (0.1 unit for 3 h at 28°C), (c) treated with pepsin (1:100, w/w, for 18 h at 4°C in 0.5 M-acetic acid/HCl, pH 2), and (d) treated with pepsin and then adjusted to pH 7.4 with 4 mM-CaCl₂/1 M-Tris before incubation with collagenase as in (b). Migration is from top (–) to bottom (+). Bands are: 1, p-N-collagen type III α_1 -chain; 2, collagen type III α_1 -chain and three-quarter N-terminal fragment of p-N-collagen type III α_1 -chain; 3, three-quarter N-terminal fragment of collagen type III α_1 -chain; 4, one-quarter C-terminal fragment of collagen type III α_1 -chain; 5, one-quarter C-terminal fragment of collagen type III α_1 -chain lacking the C-terminal telopeptide. An unfortunate cut used for identification of the plate suppressed the pattern of the β -chain and higher-molecular-weight component in sample (a).

than those shown in Fig. 1(b) and labelled. These one-quarter C-terminal peptides are known to contain two half-cystine residues per chain (Glanville *et al.*, 1976).

Incubation of the p-N-collagen type III, native (Fig. 2a) or [14 C]carboxymethylated (Fig. 3a), with the 35–20% saturation-(NH $_4$) $_2$ SO $_4$ fraction of fibroblast culture medium resulted in the conversion of p-N-collagen type III α_1 -chains into collagen type III α_1 -chains in stoichiometric amount (Fig. 2b) and the generation of a labelled fragment (Fig. 3e). Under conditions of the assay similar to those described for p-N-collagen type I peptidase (Nusgens & Lapière, 1979), the uncleaved p-N-collagen type III and collagen type III were precipitated by 27% (v/v) ethanol (Figs. 3b and 3f). The excised fragment remaining in 27%-ethanol solution was precipitated by adding ethanol to 70% (v/v) (Fig. 3g). The absence of label from the 70%-ethanol supernatant demonstrated the lack of unspecific proteolysis (Fig. 3h). Measurement of the radioactivity released between 27 and 70% ethanol represented therefore a measure of the generation of the N-terminal peptide from p-N-collagen type III. One unit of p-N-collagen type III peptidase was defined as the amount of activity that released all of the N-terminal peptide of 1 μ g of p-N-collagen type III/h at 26°C.

One has to note on the disc-gel electrophoretogram (seen also on the fluorogram, but less distinctly) that the pattern of the dimers (in Figs. 2a and 2b) was modified by incubation in the presence

Fig. 3. Fluorogram of [14 C]carboxymethylated p-N-collagen type III incubated without and with p-N-collagen type III peptidase

After incubation of 30 μ g of [14 C]carboxymethylated p-N-collagen type III in the absence (a–d) and in the presence (e–h) of 2 units of p-N-collagen type III peptidase at 26°C for 16 h, a portion of the incubation mixture was freeze-dried and electrophoresed on a polyacrylamide slab gel (12.5% acrylamide) containing 4 M-urea in the presence of dithiothreitol (tracks a and e). The remaining incubation mixture was adjusted to 27% (v/v) ethanol, and the resulting precipitate (tracks b and f) was electrophoresed as above in a polyacrylamide gel (12.5% acrylamide). The 27%-ethanol supernatant was adjusted to 70% (v/v) ethanol. The precipitate (tracks c and g) and supernatant (tracks d and h) were freeze-dried and electrophoresed as above. The labelled proteins were detected by fluorography and exposure to a Kodak Royal X-O-mat film.

Fig. 2. Densitometric recording of SDS/polyacrylamide-gel electrophoretogram of native p-N-collagen type III after incubation without and with p-N-collagen type III peptidase

After incubation of 15 μ g of p-N-collagen type III at 26°C for 16 h in the absence (a) and in the presence (b) of 0.7 unit of p-N-collagen type III peptidase, the reaction mixture was adjusted to 27% (v/v) ethanol. The precipitate was collected by centrifugation and denatured in 10 mM-Tris/HCl buffer, pH 6.8, containing 4 M-urea, 2% (w/v) SDS, 10% (w/v) sucrose, 50 mM-dithiothreitol and 0.05% Bromophenol Blue at 60°C for 10 min. Disc-gel electrophoresis was performed on polyacrylamide gel (7.5% acrylamide) containing 4 M-urea and 1% SDS at 5 mA/tube. Migration is from right to left. The bands stained by Coomassie Blue were recorded with a Densicord densitometer.

of p-N-collagen type III peptidase, although the proportion of collagen β -chains to p-N-collagen α -chains + collagen α -chains is unchanged. It suggests the existence of dimers containing p-N-collagen type III α_1 -chains that are converted into dimers containing collagen type III α_1 -chains without excision of the peptide region (telopeptide) that links together the two monomers.

There was no detectable p-N-collagen type III peptidase activity in the unfractionated fibroblast culture medium. The p-N-collagen type III peptidase activity was only detected in the fraction precipitated at 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ or its sub-fractions at 40–50%, 35–20%, 20–15% and 15–0% saturation with $(\text{NH}_4)_2\text{SO}_4$. The most active fraction (35–20% saturation) yielded, on filtration on Ultrogel AcA-34, two peaks of activity of molecular size 180 000 and 100 000, human immunoglobulin G (150 000) and transferrin (77 000) being used for calibration. A significant proportion of activity of each peak was retained on concanavalin A-Sepharose and eluted by methyl α -D-mannoside (results not shown).

The partly purified enzyme behaved as an endopeptidase against p-N-collagen type III and carboxymethylated p-N-collagen type III, both native and heat-denatured. It did not display peptidase activity on p-N-collagen type I or its gelatin.

The cleavage site was estimated to lie between the telopeptide and the helical part of the p-N peptide. This was supported by the molecular weight of the [^{14}C]carboxymethylated products of cleavage of p-N-collagen type III peptidase compared with those obtained by bacterial collagenase (Fig. 4). In the absence of reduction before electrophoresis (Figs. 4a, 4b and 4c), the p-N peptide obtained by reaction with p-N-collagen type III peptidase (Fig. 4a) migrated at a position similar to that of the p-N peptide released from p-N-collagen type III by bacterial collagenase (Fig. 4c). When reduction in denaturing conditions was performed before electrophoresis (Figs. 4a', 4b' and 4c'), the migration of both products was again similar, but faster than in the absence of reduction. The molecular size of the reduced p-N peptide from p-N-collagen type III was apparently larger than that of the p-N peptide (Fig. 4d) resulting from the cleavage of p-N-collagen type I by p-N-collagen type I peptidase.

The 35–20%-saturation- $(\text{NH}_4)_2\text{SO}_4$ fraction was used for kinetic studies and further characterization. The generation of p-N peptide was proportional to the amount of enzyme solution up to 50% cleavage of the substrate (Fig. 5a) and within these limits to the time of reaction (results not shown). We determined an apparent K_m for carboxymethylated p-N-collagen III (Fig. 5b) that was found to be $0.3 \times 10^{-6}\text{M}$, one order of magnitude lower than that

Fig. 4. Comparison of molecular size of p-N peptides obtained by cleavage of p-N-collagen type III with p-N-collagen type III peptidase and bacterial collagenase

Purified p-N-collagen type III was incubated at 26°C for 16 h in the presence of p-N-collagen type III peptidase or collagenase. p-N-collagen type III peptidase alone was treated under the same conditions as a control. The mixtures were adjusted to 27% (v/v) ethanol, the precipitate was discarded and the supernatant was freeze-dried. The freeze-dried samples were reduced with dithiothreitol and carboxymethylated with iodo[^{14}C]acetamide. The labelled peptides were electrophoresed after denaturation at 60°C in the presence and in the absence of dithiothreitol in a polyacrylamide gel (12.5% acrylamide) and detected by fluorography under the same conditions as indicated in Fig. 2 legend except that urea was omitted. Tracks (a) and (a'), p-N-collagen type III peptidase-cleaved peptide (arrow); wells (b) and (b'), p-N-collagen type III peptidase alone; wells (c) and (c'), collagenase-cleaved peptide; well (d), p-N-collagen type I peptidase-generated peptide from p-N-collagen type I, for comparison.

reported for p-N-collagen type I peptidase ($2.8 \times 10^{-6}\text{M}$) (Nusgens & Lapière, 1979). The activity as a function of pH between pH 5.0 and 9.0 was similar for both peptidases, with a maximum for p-N-collagen type I peptidase at pH 7.2 and for p-N-collagen type III peptidase at pH 7.6. The effects of various metal ions on the activity of the two peptidases were also similar. Both enzymes were inhibited to the same extent by 0.2mM- Cu^{2+} (50%) and 0.2mM- Zn^{2+} (40%) and not by 0.2mM- Co^{2+} , - Mg^{2+} or - Mn^{2+} . Phenylmethanesulphonyl fluoride and N-ethylmaleimide did not inhibit the procollagen peptidase. Whereas p-N-collagen type I peptidase extracted from tissue (skin or tendon) was irreversibly inhibited by chelating Ca^{2+} with EDTA, p-N-collagen type III peptidase from tendon fibro-

Fig. 5. Kinetic parameters of the enzymic reaction catalysed by *p*-N-collagen type III peptidase

(a) Dependence of activity on enzyme concentration; 30 μg of *p*-N-collagen type III is incubated with increasing amounts of enzyme solution (1 mg of protein/ml) under the standard conditions described in the Materials and methods section. Enzyme activity is expressed as the radioactivity precipitated by ethanol between 27 and 70% (v/v) (radioactivity released) after 16 h of incubation at 26°C. (b) Double-reciprocal plot of initial velocity against increasing concentration of radiolabelled substrate for two different amounts of enzyme, 15 μg (O) and 25 μg (●) of protein [fraction precipitated between 35% and 20% saturation with $(\text{NH}_4)_2\text{SO}_4$]. Velocity (v) is expressed in pmol of *p*-N-collagen type III α_1 -chain converted into collagen type III α_1 -chain/h. The substrate concentration $[S]$ is expressed in μmol of *p*-N-collagen type III α_1 -chain/litre.

Fig. 6. *p*-N-collagen type III peptidase and total collagen synthesis as a function of confluency in tendon fibroblast culture

p-N-collagen type III peptidase activity in units/dish was measured in various $(\text{NH}_4)_2\text{SO}_4$ -saturation fractions (▨, 15–0% saturation; ■, 20–15% saturation; □, 35–20% saturation; ▤, 40–50% saturation) collected from the medium of normal tendon fibroblasts at increasing times after confluency (days). Collagen biosynthesis (●) was measured in a 20%-(v/v)-ethanol precipitate of similar culture media after 24 h of labelling with $[2,3\text{-}^3\text{H}]\text{proline}$ (5 $\mu\text{Ci/ml}$) and determination of hydroxyproline by the technique of Juva & Prockop (1966).

blast culture was not affected by this reagent. *p*-N-collagen type III peptidase was irreversibly inhibited by dithiothreitol. Preincubation of the enzyme with dithiothreitol followed by the addition of an excess of *N*-ethylmaleimide did not allow recovery of the activity, but *N*-ethylmaleimide in slight excess of dithiothreitol in the reaction mixture allowed complete conservation of this activity.

The *p*-N-collagen type III peptidase activity in the medium of tendon fibroblasts in culture was studied as function of the time elapsed from the plating of the cells. The activity present in the medium paralleled the synthesis of collagen (Fig. 6); similar results were obtained for the production of *p*-N-collagen type I peptidase (B. V. Nusgens & Ch. M. Lapière, unpublished work).

Discussion

p-N-collagen type III peptidase isolated from the spent medium of cultured tendon fibroblasts can be described as a specific endopeptidase excising in a single fragment the *N*-terminal extension of *p*-N-collagen type III. The specificity of the peptidase is supported by its lack of activity on the very long and fragile polypeptide of the type I gelatin and its restricted endopeptidase activity on its substrate *p*-N-collagen type III.

The molecular size of the products of lysis of *p*-N-collagen type III by *p*-N-collagen type III peptidase suggests that the cleaved peptide bond lies

in a well-defined region of the N-terminal extension at the junction with the telopeptide. The p-N peptides cleaved by p-N-collagen type III peptidase and that resulting from digestion by bacterial collagenase are indeed comparable in size. Under the condition of the assay, bacterial collagenase should indeed cleave the collagen α -chain without affecting the N-terminal non-helical extension. It is known that the presence of disulphide bridges in or close to the helical 'collagen-like' portion of the N-terminal extension of p-N-collagen type III makes it resistant to bacterial collagenase, and that such disulphide bridges require reduction under highly denaturing conditions in order for their influence to be suppressed (Brückner *et al.*, 1978). In the absence of reduction before electrophoresis, the peptidase-generated and collagenase-generated peptides are indeed in the form of a polymer, probably a trimer. On reduction at high temperature (60°C) in urea before electrophoresis, both dissociate into monomers. Before or after reduction, the peptidase-derived peptide displays a slightly faster migration in a 12%-acrylamide polyacrylamide gel than does the collagenase-generated peptide. This small difference in the electrophoretic mobility can be accounted for by the 20 amino acids forming the telopeptide present in the collagenase-derived peptide and absent from the peptide released by p-N-collagen type III peptidase.

The easily reducible disulphide bridges at the N-terminal part of the extension have no significant function in maintaining the ultrastructure required for endopeptidase activity. p-N-collagen type III peptidase as well as p-N-collagen type I peptidase are indeed active on their respective substrates after carboxymethylation. We (Kohn *et al.*, 1974) and others (Tuderman *et al.*, 1978) have observed an extremely diminished activity of p-N-collagen type I peptidase when its substrate is denatured, whereas p-N-collagen type III peptidase displays an unmodified activity on denatured carboxymethylated p-N-collagen type III. The presence of reduction-resistant disulphide bridges leading to a fast renaturation of the helical section of the N-terminal extension of p-N-collagen type III (Brückner *et al.*, 1978) could explain this difference. It suggests that the structural integrity of the helical section of the extension is required for enzyme activity. It might represent the binding site of the enzymes.

The irreversible inhibition of the procollagen peptidases by reducing agents such as dithiothreitol can be explained by two processes. It could be related either to an unfolding of the binding site or the active site of the peptidase or to the dissociation of an enzyme composed of subunits maintained by disulphide bridges. Additional work is required to resolve the issue.

It seems certain that p-N-collagen type I pepti-

dase and p-N-collagen type III peptidase are different proteins. Beside differences in substrate specificity (p-N-collagen type III peptidase is inactive against p-N-collagen type I, and vice versa) and affinity for their respective substrates (one order of magnitude higher for p-N-collagen type III peptidase as compared with p-N-collagen type I peptidase), the two peptidases differ also in terms of metal-ion requirements (tissue-extracted p-N-collagen type I peptidase is inhibited by EDTA; Lapière *et al.*, 1971) and susceptibility to inhibition by serum proteins (H. Shinkai, B. V. Nusgens & Ch. M. Lapière, unpublished work). The apparent molecular sizes of the two endopeptidases are similar, but, whereas p-N-collagen type I peptidase displays an apparent mol.wt. 200 000 by filtration on Ultrogel AcA-34, p-N-collagen type III peptidase is eluted in two fractions, one of mol.wt. 180 000–150 000 and the other of mol.wt. 100 000. As reported by Tuderman *et al.* (1978) and also observed by us (H. Shinkai, B. V. Nusgens & Ch. M. Lapière, unpublished work), p-N-collagen type I peptidase is a glycoprotein that displays a high affinity for concanavalin A–Sepharose. p-N-collagen type III peptidase of either molecular size was only partly bound to that lectin, and no obvious difference in activity was observed between the retained and the unbound fraction. Some of these differences might be related to the source of enzyme, tissues for p-N-collagen type I peptidase and cells in culture for p-N-collagen type III peptidase, since this could result in different types of proteins, precursors or enzyme–inhibitor complexes, as discussed for collagenase (Woolley & Evanson, 1977). The best evidence for a difference between the two endopeptidases is the existence of an antibody against p-N-collagen type I peptidase, raised in the rabbit (H. Shinkai, B. V. Nusgens & Ch. M. Lapière, unpublished work), that suppresses the activity of this endopeptidase, but does not react with p-N-collagen type III peptidase and does not alter the activity of that endopeptidase when added to the reaction mixture.

It might seem curious to collect p-N-collagen type III peptidase from cultured tendon fibroblasts, because tendon is known to contain only trace amounts of collagen type III and of p-N-collagen type III peptidase (B. V. Nusgens & Ch. M. Lapière, unpublished work). The production of p-N-collagen type III peptidase occurs only after several subcultures of tendon fibroblasts and seems to represent, together with the synthesis of type III collagen by these cells (C. E. De Rycker, B. V. Nusgens & Ch. M. Lapière, unpublished work), an alteration in the phenotypic expression of the cells when cultured *in vitro*.

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