Purification and characterization of the *N*-terminal propeptide of human type III procollagen

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The *N*-terminal propeptide of type III procollagen was purified from human ascitic fluid by using $(\text{NH}_4)_2\text{SO}_4$ precipitation, DEAE-Sephacel chromatography at pH 8.6, Sephacryl S-300 chromatography and another DEAE-Sephacel chromatography at pH 4.5. The M_r of the human peptide was about 42000, which corresponds in size to the propeptide released by the specific *N*-proteinase during the extracellular processing of collagen. Bacterial-collagenase digestion of the human peptide produced three fragments, which could be separated on a Bio-Gel P-10 column. The human propeptide and its collagenase-derived fragments, an *N*-terminal non-collagenous domain Col 1, a *C*-terminal non-helical domain Col 2 and a collagenous domain Col 3, resembled those derived from the *N*-terminal segment of bovine type III procollagen in their amino acid composition. The human peptide was found to contain sulphate, which may explain its extremely low isoelectric point (3.1). Antibodies against the human *N*-terminal propeptide reacted similarly with both the purified human peptide and a corresponding segment of bovine type III procollagen. The human propeptide could be used in developing radioimmunoassays for monitoring fibrotic processes.

INTRODUCTION

The interstitial collagens (types I to III) are synthesized in the form of larger molecules, procollagens, which contain additional precursor-specific peptide extensions at both the *N*-terminal and the *C*-terminal ends (Prockop *et al.*, 1979*a,b*). During the conversion of procollagen into collagen, these extensions are removed by specific proteinases (Fessler & Fessler, 1978), a conversion that appears to be necessary for the formation of native-type collagen fibrils (Miyahara *et al.*, 1984). It has been suggested that the *N*-terminal propeptides liberated may act as feedback regulators for collagen biosynthesis (Wiestner *et al.*, 1979; Paglia *et al.*, 1981). Conversion seems to be a slow process, however, since collagen molecules that still contain the *N*-terminal extensions (pN-collagens) can be found in tissues (Gay *et al.*, 1975; Wick *et al.*, 1978; Fleischmajer *et al.*, 1981).

Type III pN-collagen has been purified from lathyrus rat skin (Byers *et al.*, 1974; Lenaers & Lapiere, 1977) and foetal bovine skin (Timpl *et al.*, 1975), and the *N*-terminal segment has been isolated after bacterial-collagenase digestion of this material (Nowack *et al.*, 1976). The extension peptide obtained is larger than the propeptide produced by the cleaving enzyme *in vivo*, since it also contains the non-collagenous telopeptide region of the collagen molecule proper. The *N*-terminal propeptide contains a large globular region (domain Col 1), a short collagenous triple helix (domain Col 3) and a non-collagenous part (domain Col 2), which connects the propeptide to the collagen molecule and which in the case of the bovine *N*-terminal segment isolated after collagenase digestion also includes the procollagen *N*-proteinase cleavage site. The disulphide bridges in the Col 2 domain are located between the three constituent chains, whereas

in the Col 1 domain these linkages only occur within the individual chains (Nowack *et al.*, 1976). At present no data are available on the purification and characterization of the *N*-terminal propeptide of type III procollagen from any human source.

Assays of the amount of the *N*-terminal propeptide of type III procollagen have been shown to be useful for monitoring type III procollagen metabolism in internal organs (Rohde *et al.*, 1979). The radioimmunoassay is based on the use of the bovine segment, which cross-reacts significantly with human samples (Rohde *et al.*, 1979; Pierard *et al.*, 1984). The antigens detected exist in serum in at least three forms, however, which differ in their antigenic properties and molecular sizes (Niemelä *et al.*, 1982). In normal serum most of the antigenicity is due to a smaller peptide form, whereas under conditions where the metabolism of type III procollagen is increased the proportion of those antigen forms equal to or larger than the authentic propeptide in size is increased (Niemelä *et al.*, 1983).

The present study reports on the purification and chemical and immunological characterization of the *N*-terminal propeptide of human type III procollagen. The propeptide isolated corresponds in size to the peptide released by the specific procollagen *N*-proteinase during the extracellular processing of collagen.

EXPERIMENTAL

Materials

Ascitic fluid was collected for palliative reasons from some patients admitted to the Oulu University Central Hospital, Oulu, Finland. Before purification, small samples from each batch of fluid were tested by

Abbreviations used: Col 1, Col 2 and Col 3, collagenase-digest fragments of the *N*-terminal segment of type III procollagen.

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chromatography on a Sephacryl S-300 column and by radioimmunoassay of the fractions for bovine Col 1-3. The protein content of the samples was measured by using a protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

DEAE-Sephacel and Sephacryl S-300 were purchased from Pharmacia (Uppsala, Sweden), bacterial collagenase (grade CLSPA) was from Worthington Corp. (Freehold, NJ, U.S.A.), barium chloranilate was from Baker Chemicals (Deventer, The Netherlands) and Na¹²⁵I from The Radiochemical Centre (Amersham, Bucks., U.K.).

Purification procedure

Batches (2-5 litres) of selected ascitic fluid were treated with solid (NH₄)₂SO₄ to yield 40% saturation. The precipitated proteins were passed over a DEAE-Sephacel column (5 cm × 50 cm) equilibrated in 50 mM-Tris/HCl buffer, pH 8.6, containing 2 M-urea (deionized with Amberlite MB-3 from BDH Chemicals, Poole, Dorset, U.K.), 2 mM-EDTA, 2 mM-N-ethylmaleimide, 2 mM-p-chloromercuribenzoate, 1 mM-phenylmethanesulphonyl fluoride and 2 mM-sodium iodoacetate. The column was eluted with a linear NaCl gradient (0-0.3 M). The fractions containing the immunologically active material in the radioimmunoassay for bovine Col 1-3 were pooled, dialysed against 0.1 M-NH₄HCO₃, pH 7.9, and freeze-dried.

The protein was dissolved in 5-15 ml of phosphate-buffered saline (0.12 M-NaCl/4 mM-KH₂PO₄/12 mM-Na₂HPO₄, pH 7.2) containing 3 mM-NaN₃ and 0.04% (v/v) Tween 20, and chromatographed on a column (1.5 cm × 110 cm) of Sephacryl S-300 equilibrated in this buffer. The fractions containing the immunological activity were pooled, dialysed against 10 mM-sodium citrate/HCl buffer, pH 4.5, and re-chromatographed on a DEAE-Sephacel column equilibrated with this buffer. Those fractions containing inhibiting activity in the assay for bovine Col 1-3 were dialysed against 0.1 M-NH₄HCO₃, pH 7.9, freeze-dried and stored frozen.

Analytical procedures

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in 12% acrylamide gels, which were stained with Coomassie Brilliant Blue.

Binding to anion-exchanger was tested by adding a constant amount of partially purified N-terminal propeptide to tubes containing DEAE-Sephacel equilibrated in 10 mM-sodium citrate/HCl buffer at different pH values (range 1.8-4.0). The tubes were then mixed by gentle stirring, the gel was allowed to settle and the supernatants were assayed by the radioimmunoassay for the Col 1-3. For precise determination of the isoelectric point an N-terminal propeptide sample was focused in a sucrose gradient in an LKB Ampholine 8100 column (Haglund, 1967) with an Ampholine concentration of 1% (v/v) and a pH range of 2.5-6.

The amino acid composition of hydrolysed (6 M-HCl for 24 h at 110 °C under N₂) protein samples was determined on a Kontron Liquimat III amino acid analyser. The values for serine and threonine were corrected for losses during hydrolysis by the factors 1.21 and 1.08 respectively.

Inorganic sulphate was measured before and after acid hydrolysis (6 M-HCl, 100 °C, 4 h) of the samples by the barium chloranilate method (Spencer, 1960). The amount of covalently bound sulphate was calculated from the

total amount recovered from hydrolysed samples by subtracting the amount recovered without acid hydrolysis (20-40% of the total sulphate). Inorganic phosphate was determined by the method of Baginski *et al.* (1975). The molar proportions of monosaccharides were determined after methanolysis by g.l.c. (Bhatti *et al.*, 1970). Methanolysis was performed either directly on the N-terminal propeptide or after Pronase digestion and purification of the glycopeptides on a column of Bio-Gel P-4. Sialic acid was also determined colorimetrically (Miettinen & Takki-Luukkainen, 1959).

Preparation of the peptide fragments Col 1 and Col 2

Collagenase digestion of the N-terminal propeptide was carried out as described previously (Bruckner *et al.*, 1978). A 10 mg portion of the human peptide was dissolved in 50 mM-Tris/HCl buffer, pH 8.0, containing 20 mM-CaCl₂, 70 units of bacterial collagenase were added and the mixture was incubated at 55 °C for 10 min. After the mixture had cooled to 37 °C another 70 units of collagenase were added. Digestion was allowed to proceed for 3 h, after which the reaction mixture was applied to a Bio-Gel P-10 column.

Immunological methods

The bovine Col 1-3 was isolated from foetal calf skin and antisera were prepared in rabbits as described previously (Niemelä *et al.*, 1982). Antisera against human N-terminal propeptide were raised in rabbits by three injections of the human peptide (0.5 mg in 1 ml) dissolved in 0.1 M-phosphate buffer, pH 7.2, and mixed with an equal volume of complete Freund's adjuvant given at 4-week intervals. These antisera were absorbed on purified human type III N-terminal propeptide linked to Sepharose 4B. The propeptide was labelled with ¹²⁵I by the chloramine-T method (Timpl & Risteli, 1982). In the inhibition assay 0.2 ml of a non-labelled antigen solution was preincubated with 0.1 ml of absorbed antibodies at a dilution capable of binding 50% of the labelled antigen before the addition of labelled antigen. Antibody-antigen complexes were precipitated by goat antiserum to rabbit IgG.

RESULTS

Antigens related to the N-terminal propeptide of type III procollagen in human ascitic fluid

The ascitic fluids contained 30-60 g of protein/l, of which 3-10 mg generally reacted with the antibodies to the bovine Col 1-3. The different batches varied greatly in the proportions of the various antigen forms. Most of the propeptide antigenicity in the batch selected for purification, which had been obtained from a patient who had pancreatic carcinoma with metastases throughout the peritoneum, was eluted in the same position as the reference bovine segment (Fig. 1).

Purification of the N-terminal propeptide of human type III procollagen

The purification procedure summarized in Table 1 has so far been used for isolating the human N-terminal propeptide from eight batches of ascitic fluid obtained from one patient. After (NH₄)₂SO₄ precipitation all of the authentic N-terminal-propeptide antigenicity that was of the right propeptide size was recovered, whereas the

Fig. 1. Gel filtration on a Sephacryl S-300 column of the human ascitic fluid selected for purification

Fractions (2.5 ml) were collected and the propeptide antigenicity was identified by using a radioimmunoassay for Col 1-3. —, A_{280} ; ○, *N*-terminal propeptide. The arrow indicates the elution position of the standard bovine Col 1-3.

antigen form smaller in size than the propeptide proper mainly remained in solution.

In the DEAE-Sephacel chromatography at pH 8.6 a peak of propeptide antigenicity appeared late in the gradient. This was further chromatographed on a Sephacryl S-300 gel-filtration column, where only one peak of antigenicity was observed in the position of the reference bovine peptide (see Fig. 1). The isoelectric point of the material recovered from the gel-filtration column was about pH 3.0, when judged by binding to DEAE-Sephacel at different pH values. Thus final purification was achieved by re-chromatography on DEAE-Sephacel, now at pH 4.5. The *N*-terminal propeptide of human type III procollagen was eluted in a single peak late in a linear gradient of NaCl (Fig. 2).

Some 10–20% of the original antigenic activity was recovered after these steps, and the human propeptide was homogeneous when studied by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 3). The purity of the propeptide was also confirmed by h.p.l.c., with a reverse-phase column, where one sharp peak was seen (results not shown).

Molecular properties of the *N*-terminal propeptide of human type III procollagen

The mobility of the purified propeptide in sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was compared with that of bovine Col 1-3 (Fig. 3), which had been prepared by collagenase digestion of the whole type

Fig. 2. Chromatography of the antigenically active material obtained from the Sephacryl S-300 column on a DEAE-Sephacel column, pH 4.5

The bound proteins were eluted with a 600 ml linear gradient of 0–0.4 M-NaCl and collected into 10 ml fractions. —, A_{280} ; ○, *N*-terminal propeptide. The arrow denotes the start of the gradient and the horizontal bar the fractions containing the propeptide antigenicity.

III procollagen molecule. The human propeptide was apparently some 3000 daltons smaller than the reference bovine segment when compared with standard globular proteins. The M_r of the bovine *N*-terminal segment is 45000 (Bruckner *et al.*, 1978), and thus that of the human propeptide would be 42000. The apparent M_r of the polypeptide chains of the human propeptide after reduction was about 14000.

The amino acid composition of the human *N*-terminal propeptide (Table 2) resembles that of the bovine *N*-terminal propeptide calculated from the sequence data (Brandt *et al.*, 1984), except that the glutamic acid content is somewhat higher. The purified human propeptide was found to contain sulphate (about 10 mol/mol of *N*-terminal propeptide). Most of the sulphate was located in the globular domain Col 1 (about 2 mol/mol of monomeric chain), whereas the domains Col 2 and Col 3 contained no covalently bound sulphate. No phosphate and only traces of different monosaccharides (1 mol or less/mol of *N*-terminal propeptide) were found. After isoelectric focusing, the *N*-terminal propeptide was found in the fraction of pH 3.1, which is in good agreement with the results of binding to DEAE-Sephacel.

Defined fragments of the *N*-terminal propeptide of type III procollagen

The human *N*-terminal propeptide was treated with bacterial collagenase after denaturation at 55 °C, and the

Table 1. Purification of the *N*-terminal propeptide of human type III procollagen

For full experimental details see the text.

	Protein (mg)	<i>N</i> -terminal propeptide (mg)	Recovery (%)	Purification (fold)
Starting material	142000	19.5	100	1
(NH ₄) ₂ SO ₄ precipitate	42100	19.5	100	3
DEAE-Sephacel, pH 8.6	69	9.0	46	960
Sephacryl S-300	18	7.5	38	3070
DEAE-Sephacel, pH 4.5	3.7	3.7	19	7360

Fig. 3. Slab-gel electrophoresis of human *N*-terminal propeptide (b) and bovine Col 1–3 (M_r 45000) (a)

The standard globular proteins shown are phosphorylase *b* (M_r 94000), albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000) and trypsin inhibitor (M_r 20100) ('low-molecular-weight' standards; Pharmacia). Overloading of the gel did not reveal impurities.

collagenase digest was resolved into three peaks by chromatography on a Bio-Gel P-10 gel-filtration column (Fig. 4). The two peaks that were eluted near the void volume of the column, Col 1 and Col 2, had non-collagenous amino acid compositions (Table 2), whereas the material that was eluted in very small peptide fragments (Col 3; Fig. 4) was typically collagenous in composition (Table 2).

Immunochemical properties of the *N*-terminal propeptide of human type III procollagen

A rabbit antiserum was prepared against the human *N*-terminal propeptide and the antibodies were rendered specific by affinity chromatography on this peptide coupled to Sepharose 4B. The antibodies were found to bind the labelled human propeptide in a radioimmunoassay, and a 1:8000 dilution of the antibodies, binding 50% of 1 ng of the labelled propeptide, was selected for use in the inhibition tests. Less than 1% of the label was precipitated in the presence of non-immune rabbit serum.

In a radioimmuno-inhibition assay 50% of the label was displaced by about 30 pg of the non-labelled peptide (Fig. 5). The reaction between the labelled human propeptide and its antibody was inhibited by similar amounts of

Table 2. Amino acid compositions of human *N*-terminal propeptide and its collagenase fragments

The values for human *N*-terminal propeptide and its fragments were calculated per one chain (monomer) of the trimeric *N*-terminal propeptide. The values for the bovine *N*-terminal segments were calculated from the sequence data (Brandt *et al.*, 1984). Abbreviation: N.D., not determined.

Amino acid	Amino acid composition (residues/molecule)				
	Bovine	Human	Col 1	Col 2	Col 3
Hyp	9	8			8
Asp	15	13	9	1	3
Thr	4	5	4	1	
Ser	8	9	5	2	3
Glu	15	21	15	2	2
Pro	18	15	10	2	4
Gly	21	19	9	2	11
Ala	4	5	3		1
Val	5	5	4		
Met					
Ile	6	5	2	1	2
Leu	3	4	3		1
Tyr	2	2	1	1	
Phe	1	2	1		
Hyl					0.5
His	1	1	1		
Lys	2	3	2		0.5
Arg	3	4	2		2
Cys	12	13	10	2	
Trp	1	N.D.

Fig. 4. Separation on Bio-Gel P-10 of the domains Col 1, Col 2 and Col 3 obtained from a bacterial-collagenase digest of denatured (at 55 °C) human *N*-terminal propeptide

The column was equilibrated in 0.1 M-NH₄HCO₃, pH 7.9. The column effluent was monitored by its absorbance at 230 nm.

the bovine *N*-terminal segment, the slopes of the inhibition curves being the same (Fig. 5). Almost complete inhibition was also achieved by the fragment Col 1 derived from the human propeptide, but the slope of the inhibition curve was less steep. The fragment Col 2 only gave slight inhibition when used in 1000-fold larger amounts than the Col 1 fragment.

Fig. 5. Radioimmunoassay with an antiserum against the human type III *N*-terminal propeptide and ^{125}I -labelled human type III *N*-terminal propeptide

The inhibitors of the reaction were human type III *N*-terminal propeptide (○), bovine Col 1-3 (●) and the fragments Col 1 (×) and Col 2 (□) of the human type III *N*-terminal propeptide.

DISCUSSION

This work, apart from being the first report on the properties of the human peptide, also marks the first isolation of the *N*-terminal propeptide of type III procollagen as a genuine product of cleavage *in vivo*, which is smaller in size than the reference bovine segment isolated after bacterial-collagenase digestion of foetal calf skin pN-collagen (Timpl *et al.*, 1975; Nowack *et al.*, 1976). Ascitic fluid proved to be the only human source available in sufficient quantities for the purification procedure, but there were large differences between the fluids from different donors in both concentration and distribution among the different antigenic forms related to the *N*-terminal propeptide of type III procollagen (Niemelä *et al.*, 1982). Choice of an ascitic fluid with minimal amounts of the antigen forms that are clearly larger or smaller than that corresponding in size to the authentic *N*-terminal propeptide is essential for efficient isolation. Because of cross-reaction, purification could be followed by radioimmunoassay for the *N*-terminal segment of bovine type III procollagen.

The human *N*-terminal propeptide was remarkably similar to the bovine segment with respect to a three-domain structure (Bruckner *et al.*, 1978). Also, the amino acid compositions of the human propeptide and its domains were similar to those of the bovine propeptide as deduced from the bovine *N*-terminal segment (Bruckner *et al.*, 1978; Brandt *et al.*, 1984). The difference in size between the human propeptide and the bovine segment would be in agreement with the size of the non-helical telopeptide region of the collagen molecule proper.

The isoelectric point of the human propeptide was tested by two methods and found to be exceptionally low. This cannot be explained on the basis of its amino acid composition, but acidic carbohydrate moieties attached to the molecule could be responsible for this acidity. The propeptides of procollagen types I and II contain asparagine-linked carbohydrate units (Clark, 1979; Guzman *et al.*, 1978). Also, it has been suggested that the amino acid residues nos. 127-129 in the sequence of the *N*-terminal propeptide of bovine type III procollagen represent a possible oligosaccharide-attachment site

(Shinkai & Lapiere, 1983; Brandt *et al.*, 1984). However, in the present study only traces of carbohydrate were found, suggesting that the *N*-terminal propeptide of human type III procollagen is not glycosylated. The possible variation in glycosylation was not tested, since the analyses were carried out with purified materials originally derived from ascitic fluid of one patient.

The presence of sulphate, reported here for the first time for the propeptides, could explain the acidic nature of the peptide. The sulphate is mainly located in the domain Col 1, but the nature of the sulphated residues is not known. However, sulphation of tyrosine residues has been suggested to be a widespread modification of proteins (Huttner, 1982). It is possible that a few tyrosine residues in the *N*-terminal propeptide are sulphated. With cultured human fibroblasts we have found that type III procollagen synthesized is labelled with radioactive sulphate, and that the label can be located to the *N*-terminal segment of the procollagen by bacterial-collagenase digestion studies (A. Jukkola, J. Risteli, O. Niemelä & L. Risteli, unpublished work).

Antibodies produced in rabbits against the purified human propeptide reacted equally well with the *N*-terminal segment of bovine type III procollagen. The major antigenic determinant was located to the domain Col 1. The sequential antigenic determinants of the bovine antigen are also located in the domain Col 1 (Rohde *et al.*, 1983; Brandt *et al.*, 1984). The immunological similarity between the bovine *N*-terminal segment and the human *N*-terminal propeptide is reflected in the fact that they give identical inhibition slopes in the radioimmunoassay for the *N*-terminal segment of bovine type III procollagen (Niemelä *et al.*, 1982). The peptides from these two species were also found here to be very similar quantitatively, since the inhibition curves, when assessed by either the human or the bovine propeptide radioimmunoassay, are practically identical.

The radioimmunoassay for the *N*-terminal propeptide of human type III procollagen could be used for monitoring fibrotic processes in humans (Niemelä, 1985). In addition, the isolated propeptides could be used in studies on the various biological functions proposed for these peptides, such as feedback inhibition of collagen biosynthesis (Wiestner *et al.*, 1979; Paglia *et al.*, 1981; Sear *et al.*, 1984).

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