Neutral Protease Cleaving the N-Terminal Propeptide of Type III Procollagen: Partial Purification and Characterization of the Enzyme from Smooth Muscle Cells of Bovine Aorta†

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ABSTRACT: Procollagen type III amino-terminal protease was detected in cultures of smooth muscle cells of fetal calf aorta, and this protease was purified about 400-fold. Only about half of the enzyme activity was consistently attached to concanavalin A–agarose (Con A–agarose). After affinity chromatography on type III pN-collagen–Sepharose, the Con A bound fraction showed only one major band with a molecular weight of about 72000, this value corresponding well with the elution position of enzyme activity in gel filtration. The enzyme did not cleave procollagens type I or type IV, and denatured type III pN-collagen also remained uncleaved. The K_m of the enzyme activity for iodo[^{14}C]acetamide-labeled type III pN-collagen was 0.76 μM. Neutral pH and Ca^{2+} were required for maximal enzymic activity. The metal chelators ethylenediaminetetraacetic acid and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid inhibited the activity as well as did dithiothreitol, but there was only little if any inhibition by several other proteinase inhibitors tested.

In the procedure where intracellularly synthesized procollagen molecules extracellularly form collagen bundles, an important and perhaps rate-limiting step of fibril formation is the release of the amino-terminal propeptides from the procollagen molecule (Timpl et al., 1975; Byers et al., 1974; Martin et al., 1975; Grant & Jackson, 1976; Prockop et al., 1976). This process is enzymatically catalyzed, and there has been speculation that it occurs as the procollagen enters the extracellular space, or immediately thereafter (Leung et al., 1979; Fessler & Fessler, 1978). This enzyme activity has been detected in many tissues and cell cultures from various species (Kohn et al., 1974; Tuderman et al., 1978; Leung et al., 1979): a type I procollagen aminopeptidase has been purified from chicken fibroblasts by Tuderman et al. (1978), and it was also reported that this enzyme also cleaves type II procollagen but not type III procollagen (Tuderman et al., 1978; Tuderman & Prockop, 1982). Nusgens et al. (1980) have described type III aminoprocollagen peptidase activity in calf leg tendon cultures and have found that this enzyme does not cleave type I procollagen. The substrate specificity of the enzyme may be of significant importance in vivo since type I and type III collagens form different kinds of fibrils; type I forming thick bundles with high optical density and type III forming thin fibers (Lapiere et al., 1977; Fleischmajer et al., 1981). Previous reports (Tuderman et al., 1978; Nusgens & Lapiere, 1979; Morris et al., 1979; Leung et al., 1979; Nusgens et al., 1980; Tuderman & Prockop, 1982) suggest several differences in the two enzyme activities responsible for the removal of N-terminal propeptides from these two procollagen types. The enzymes probably have different modes of action and regulation mechanisms that are also distinct from carboxy-terminal proteases; e.g., in fetal calf skin, collagen types I and III carry their amino-terminal extensions in variable ratios. Furthermore, dermatosparactic animals have a genetic defect in the amino-terminal protease of type I collagen, and their skin contains large amounts of uncleaved pN-collagen[1] (Lapiere et al., 1971; Lenaers et al., 1971; Schofield & Prockop, 1973; Liechtenstein et al., 1973; Fjølstad & Helle, 1974).

There has been speculation that the N-protease is bound to the cell membrane or is localized in the extracellular space (Tuderman et al., 1978; Leung et al., 1979). However, its activity has also been found in fibroblast culture medium (Nusgens et al., 1980). In our studies, no significant enzyme activity in the media of smooth muscle cells has been observed, in contrast to extractions of the cell layer. In the present series of experiments, we have partially purified this extractable, cell layer bound enzyme activity that cleaves the N-terminal propeptide from native type III procollagen but does not accept either denatured type III procollagen or type I or IV procollagen as its substrate.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane (Tris), phenylmethanesulfonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), DL-dithiothreitol (DTT, Cleland's reagent), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), glycine, trypsin, chymotrypsin, methyl α-mannoside, and Coomassie Brilliant Blue were from Sigma Chemical co. (St. Louis, MO). Ethylenediaminetetraacetate (EDTA) (Titriplex III), dimethyl sulfoxide (Me_2SO), and various analytical chemicals were purchased from Merck (Darmstadt, BRD). Sodium cacodylate, α,α′-dipyridyl, and sodium dodecyl sulfate (SDS) were from Fluka Ag; Ultrogel AcA 44 was from LKB (France); concanavalin A–Sepharose and cyanogen bromide activated Sepharose 4B were from Pharmacia Chemicals (Uppsala, Sweden). Iodo[^{14}C]acetamide was from the Radiochemical Centre (Amersham, England), and [^{14}C]proline was from New England Nuclear (Boston, MA). Triton X-100 was from BDH Chemicals Ltd. (Poole, England), and acrylamide, 2,5-diphenyloxazole (PPO), and Kodak X-OMAT AR films were obtained from Eastman Kodak Co. (Rochester, NY). Liquid scintillation reagent (Lumagel) was purchased from Lumac (Holland). Dulbecco's modified Eagle's medium, newborn calf serum, and other tissue culture chemicals were from Gibco (England).

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[1] Abbreviations: pN-collagen, collagen precursor that contains amino-terminal propeptide but not carboxy-terminal propeptide; N-protease, enzymic activity that removes the amino-terminal propeptides from procollagen.

0006-2960/84/0423-1251$01.50/0

Preparation of Procollagen Substrate. pN-Collagen type III was extracted from fetal calf skin by repeated salt fractionation as described by Nusgens et al. (1980). The substrate was then carboxymethylated with iodo[^{14}C]acetamide as described for pN-collagen type I (Nusgens & Lapiere, 1979). The specific activity of the end product was 0.68×10^6 cpm/μmol of hydroxyproline, and the purity of the product was measured by SDS–polyacrylamide gel electrophoresis before and after chymotrypsin (1 mg/mL) and trypsin (0.1 mg/mL) digestion.

Type I procollagen from 17-day-old chicken embryo tendon fibroblasts was prepared as described previously by Uitto et al. (1981) and by Dehm & Prockop (1972). The cells were labeled with 100 μCi of [^{14}C]proline per 10^9 cells, and procollagen was purified by using DEAE-cellulose chromatography (Smith et al., 1972; Hoffman et al., 1976). The specific activity of this substrate was 0.2×10^6 cpm/μmol of hydroxyproline.

Type IV procollagen prepared from mouse EHS tumor organ cultures (Tryggvason et al., 1980) was a generous gift from Dr. Karl Tryggvason. For assay of enzyme activity against denatured pN-collagen, pN-collagen type III was denatured by reduction and alkylation as previously described by Engel et al. (1977). This denatured substrate was further heated for 5 min at 80 °C and cooled again quickly immediately prior to use to prevent the renaturation.

Assay of Enzyme Activity. The assay has been earlier described by Nusgens & Lapiere (1979). The reaction mixture (total volume of 250 μL) contained 15–20 μg of ^{14}C-carboxymethylated pN-collagen and 20–100 μL of enzyme preparation in 0.15 M NaCl, 2 mM $CaCl_2$, and 0.1 M sodium cacodylate buffer, pH 7.4, and it was incubated at 26 °C for 10 h. Under these conditions, the enzyme assay was found to be linear from 4 to 24 h. The enzyme reaction was stopped by cooling the assay tubes in an ice bath and by adding 0.1 volume of 1% SDS, 0.1 M DTT, and 0.3 M EDTA. pN-Collagen type III and type III collagen were precipitated by adding ice-cold absolute ethanol to a concentration of 27%, and type III N-propeptide was precipitated by adding ice-cold absolute ethanol to reach a concentration of 70%. After centrifugation (15000*g*, 30 min), the radioactivity from each supernatant was measured by using a Wallac liquid scintillation counter. One unit of enzymatic activity was defined here as the amount of enzyme activity present in 1 mg of original cell extract protein.

Smooth Muscle Cell Culture. Fetal calf aortas were obtained from the local slaughterhouse (Oulu, Finland). Thoracic aortas were removed, and endothelial and smooth muscle cells were separated as described previously by Macarak et al. (1977). Smooth muscle cells were then cultured to passages 6–13 in Dulbecco's modified Eagle's medium with 10% newborn calf serum, L-glutamine (290 μg/mL), ascorbate (50 μg/mL), streptomycin (100 μg/mL), and penicillin (100 units/mL).

Purification of the Enzyme. Ten to twenty dense, confluent plates (150 cm^2) of smooth muscle cell cultures (10×10^6 cells per plate) were scraped with a rubber policeman and extracted in buffer (1 mL/plate) containing 2 M KCl, 0.1% Triton X-100, and 0.1 M sodium cacodylate, pH 7.4 at 4 °C. The extract was stirred for 2 h and centrifuged at 11000*g* for 60 min, and the supernate was recovered. The pellet was resuspended in 10 mL of extraction buffer, stirred overnight, and centrifuged at 11000*g* for 60 min, and this supernate was combined with the first. The pooled supernates were dialyzed against 0.1 M KCl–0.1 M sodium cacodylate buffer, pH 7.4 at 4 °C. The sample was then passed through a 3.5-mL column of concanavalin A covalently bound to agarose, which had been equilibrated with the sample buffer. The flow rate was about 1 mL/h. After the sample passed through the column, it was recycled 1–2 times through the same column before the column was washed with 30 volumes of sample buffer and eluted with 0.3 M methyl α-D-mannoside, 1 M KCl, and 0.1 M sodium cacodylate buffer.

The optical density of the fractions was measured at 230 nm by using a Beckman GWB Model 24 spectrophotometer. The fractions of highest specific activity were pooled and dialyzed against 0.1 M KCl–0.1 M sodium cacodylate buffer, pH 7.4. The sample was then passed through a 3-mL column of type III pN-collagen coupled covalently to CNBr-activated Sepharose 4B, which was equilibrated with 0.1 M KCl–0.1 M sodium cacodylate buffer, pH 7.4. The flow rate was about 1 mL/h. After the column was washed with 10 column volumes of the equilibration buffer, the sample was eluted with 1 M KCl–0.1 M sodium cacodylate buffer, pH 7.4 at 4 °C. Fractions with eluted protein were pooled and assayed for enzyme activity.

Part of the concanavalin A eluted sample was precipitated with 2 volumes of 90% ammonium sulfate in 0.1 M sodium cacodylate buffer, the final concentration of ammonium sulfate being 60%. The sample was stirred for 2 h and centrifuged at 11000*g* for 1 h, and the pellet was recovered, dissolved in 1 M KCl and 0.1 M sodium cacodylate buffer, and dialyzed against the same buffer. The sample was then chromatographed by gel filtration on an Ultrogel AcA 44 column (1.5 × 81 cm). Fractions of 1.6 mL were collected, fractions of high optical density at 230 nm were pooled, and the enzyme activity was measured.

SDS–Polyacrylamide Slab Gel Electrophoresis. Polyacrylamide slab gel electrophoresis was carried out as described by King & Laemmli (1971). The electrophoresis was carried out at room temperature using a constant voltage of 80–100 mV. In the experiments with labeled substrate, the gel was impregnated with Me_2SO–PPO, dried under vacuum, and exposed to X-ray films (Bonner & Laskey, 1974; Laskey & Mills, 1975). When the unlabeled protein samples were studied, the gels were stained in 0.25% Coomassie Brilliant Blue in 20% trichloroacetic acid for 1 h and were then destained in 7.5% acetic acid and 15% methanol.

Protein concentrations were measured when necessary by using the Bio-Rad protein assay. The K_m value of the enzyme was measured at six different concentrations of pN-collagen and at two different concentrations of the enzyme. The K_m value was estimated from the double-reciprocal or Lineweaver–Burk plots which were analyzed with a Compucorp Scientific instrument using the linear regression analysis provided by the manufacturer.

Results

Purification of Enzyme Activity. The purification data are summarized in Table I. In the extraction with 2 M KCl and 0.1% Triton X-100 in 0.1 M sodium cacodylate buffer, pH 7.4, about 88% of the total enzyme activity found in the cell homogenate directly was detected in the cell extract. Extraction with buffered 2 M KCl alone was not as effective, only 15–20% of the activity being extractable.

The enzyme preparation was then passed through a concanavalin A–Sepharose column in 0.1 M KCl–0.1 M sodium cacodylate buffer (Figure 1). Only a part of the enzyme activity became attached to Con A–Sepharose and could be eluted with 0.3 M methyl α-mannoside in 1 M KCl–0.1 M sodium cacodylate buffer (fraction I); another pool of the

Table I: Purification of Procollagen Type III N-Protease[a]

	total protein (mg)	sp act. (units/mg)	recovery of activity (% initial)
original, lysed cells	509	1	100
cell extract	257.4	1.79	88
Con A eluted (fraction I)	13.5	10.53	27
pN III-Sepharose (fraction I)	0.099	387	7.3
breakthrough of Con A (fraction II)	151.8	2.8	52
pN III-Sepharose (fraction II)	0.028	53	0.28

[a] The protein concentration in smooth muscle cell extracts homogenized by ultrasound was measured by the method of Lowry et al. (1951), as modified by Dulley & Grieve (1975). Other samples were assayed by measuring their optical density at 230 nm and by using the Bio-Rad protein assay.

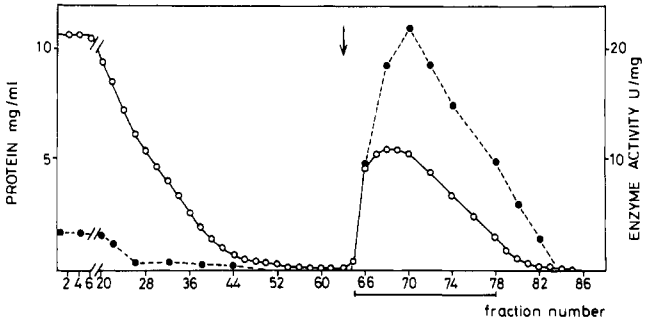

FIGURE 1: Chromatography of procollagen type III N-protease activity on a concanavalin A–agarose column. The original tissue extract was dialyzed against 0.1 M KCl and 0.1 M sodium cacodylate buffer, pH 7.4, and passed through the column with a flow rate of 1–2 mL/h. The column was washed first with the buffer and then eluted with 1 M KCl–0.1 M sodium cacodylate buffer, pH 7.4, containing 0.3 M methyl α-D-mannoside (arrow). Fractions of 1.5 mL were collected. The enzyme activity was consistently eluted in the same fractions as the other attached proteins. The elution peak with high optical density was thus routinely pooled. (○) Protein concentration; (●) enzymatic activity.

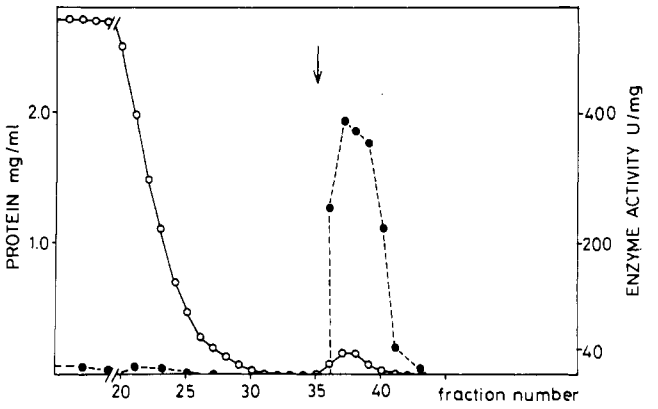

FIGURE 2: Chromatography of N-protease activity on a type III pN-collagen affinity column as described in the text. Fractions of 2 mL were pooled. (○) Protein concentration; (●) enzymatic activity; the arrow indicates the initiation of elution with 1 M KCl in 0.1 M sodium cacodylate buffer.

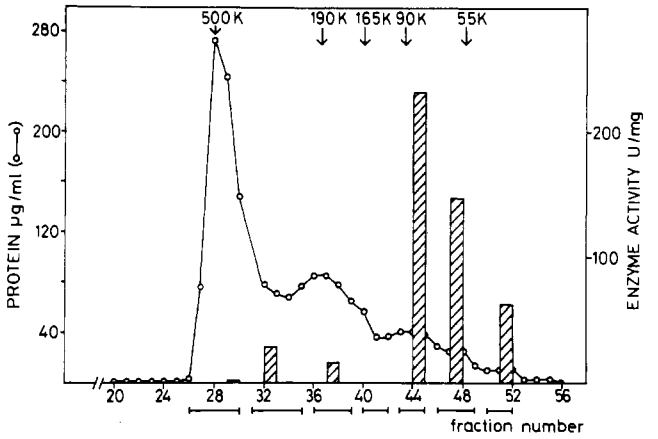

FIGURE 3: Gel filtration of procollagen type III N-protease activity on Ultrogel AcA-44 as described in the text. Arrows indicate the elution volumes of the molecular weight standards. Fractions of 1.6 mL were collected, and the protein peaks were pooled and precipitated with ammonium sulfate before the enzyme activity was measured. (○) Protein concentration; the striped boxes indicate enzymatic activity.

enzyme activity had no affinity for Con A–Sepharose (fraction II). If this breakthrough pool of enzyme activity was passed through the column repetitively, no detectable enzyme activity was attached to the column. Enzyme fractions I and II were separately pooled and passed onto the type III pN-collagen affinity column. Both fractions of enzyme activity became attached to this affinity column in the presence of 0.1 M KCl–0.1 M sodium cacodylate buffer, pH 7.4, and could be identically eluted with 1 M KCl in cacodylate buffer (see Figure 2). No detectable enzyme activity was found in the breakthrough volume of the pN III affinity column in the case of either fraction. The only difference between the two enzyme fractions was that 0.73% of the total proteins from fraction I became attached to the pN III affinity column, this amount being only 0.02% in the case of fraction II (see Table I). When the two enzyme fractions were passed through Ultrogel AcA 44, the fraction eluted from the Con A–Sepharose column (fraction I) showed the highest specific activity in elution volume, corresponding to a molecular weight of about 75 000 (see Figure 3). Because of very small quantities and inactivation of enzyme fraction II, we were not able to demonstrate its elution position by using gel filtration. In agreement with it's chromatographic elution position, fraction I repetitively produced one major protein band in SDS electrophoresis corresponding to a molecular weight of 72 000 although some smaller proteins could be seen close to the dye front. SDS electrophoresis of fraction II produced two major bands with molecular weights of 72 000 and 145 000, but the M_r 145 000 band was considerably more dominant.

Preliminary Characterization of Enzyme Activity. All the characterization tests were performed with enzyme fraction I. As previously described (Byers et al., 1974; Timpl et al., 1975; Lenaers & Lapiere, 1976; Nusgens et al., 1980), the polypeptide extension of pN-collagen type III, containing most of the cystine residues, is located at the amino-terminal end of the molecule. However, type III collagen molecules also have cystine residues at the carboxy-terminal end of the helix (Miller, 1973; Eyre, 1980). In our labeled substrate, the radioactivity was therefore also located, except in the pN type III chains, in type III collagen chains after trypsin–chymotrypsin digestion. Sequential precipitation with 27% and 70% ethanol, according to Nusgens et al. (1980), was therefore used to confirm the specificity of the assay, since free amino-terminal propeptides coprecipitate with small molecules in 70% ethanol and no labeled molecules with corresponding molecular weights were detected in SDS electrophoresis of 27% precipitated material. In our experiments, only a constant 9–10% of the total radioactivity was found in the supernatant after 70% precipitation, as evidence of some unspecific protein cleavage during the incubation period.

Incubation of labeled pN-collagen type III with the enzyme preparation resulted in the conversion of pN-collagen type III

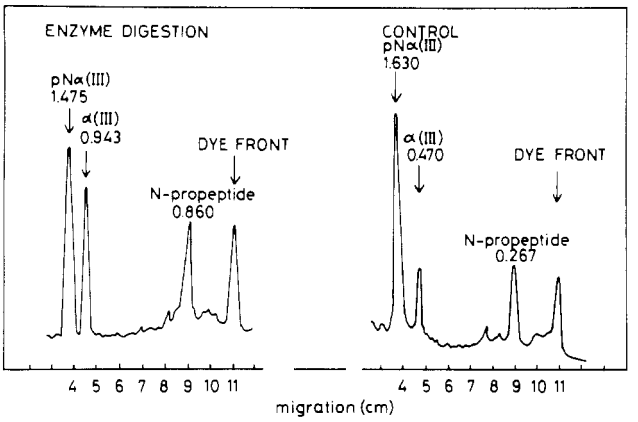

FIGURE 4: Analysis of type III pN-collagen reaction products. 20–30 μg of type III pN-collagen was incubated with 10 units of type III procollagen N-protease. The whole reaction mixture was studied. Electrophoresis was run on a 4–10% polyacrylamide gel gradient with 2 M urea after reduction with 10% β-mercaptoethanol. Fluorograms of the gels were scanned with a densitometer, and the areas of each peak were measured by a surface analyzer (Spectra-Physics SP 4100 computing integrator). The migration position of the N-propeptide is based on comparison with the migration of purified N-propeptide of pN type III collagen from fetal calf skin according to Engel et al. (1977) and was a kind gift from Dr. Leila Risteli, Department of Medical Biochemistry, University of Oulu, Finland. This propeptide achieved by collagenase digestion has a molecular weight slightly higher than that of the expected end product of the cleavage by N-protease.

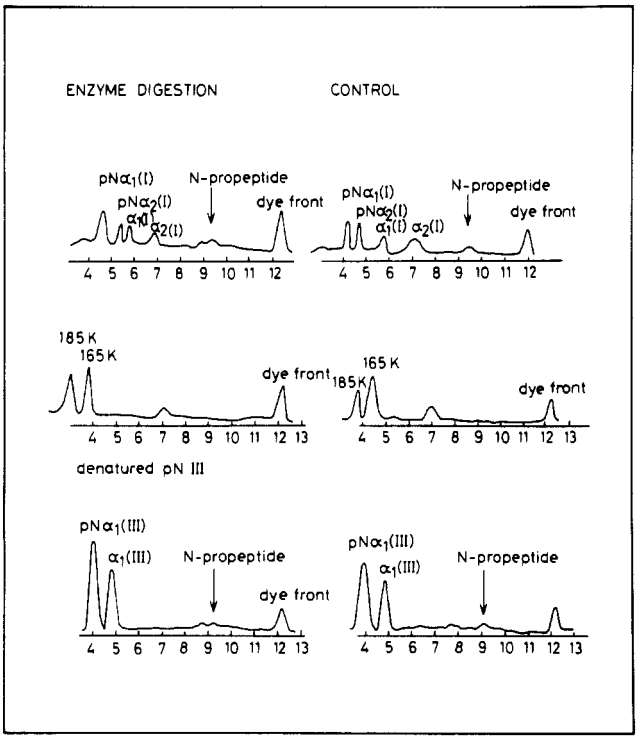

FIGURE 5: Analysis of type I pN-collagen, type IV procollagen, and denatured type III pN-collagen reaction products after enzyme digestion. The whole reaction mixture was studied. Enzyme assays and polyacrylamide slag gels were performed as described in the text.

chains into collagen chains. Upon electrophoresis, an increase of the band comigrating with purified type III N-propeptide was also visible, and it was very clear when scanning the surface areas (Figure 4).

The enzyme appeared to be specific for native type III procollagen. If pN-collagen type III was denatured by reduction and alkylation and by heating (see Materials and Methods), no enzymatic cleavage was seen either in the assay or in SDS electrophoresis. In addition, types I and IV pro-

Table II: Effect of Metals, Metal Chelators, and Protease Inhibitors on Enzyme Activity

compd added	concn (mM)	activity (%)
metal chelators		
EDTA [a]	2	30
	20	10
EGTA [a]	2	57
α,α′-dipyridyl	0.2	100
	2	100
metals		
$CaCl_2$		36
	2	100
	4	80
	8	50
$ZnCl_2$	0.02	77
	0.2	55
	2	49
$CuCl_2$	0.02	48
	0.2	40
	2	33
$MgCl_2$	0.02	63
	0.2	60
	2	46
$MnCl_2$	0.02	60
	0.2	58
	2	54
inhibitors		
PMSF	0.1	100
	1	100
DTT	0.02	0
	0.2	12
	2	10
NEM	10	100
	50	100
others		
lysine	50	82
	200	80
calf serum	4 [b]	100
	10 [b]	150

[a] EDTA and EGTA were preincubated with the enzyme for 1 h at 30 °C before the substrate was added to the incubation mixture. [b] These values are given in percent.

collagen molecules were not cleaved by the enzyme. No radioactivity that differed from control incubations was measured after 27–70% ethanol precipitation, and the results were also confirmed by SDS–polyacrylamide slab gel electrophoresis (Figure 5).

The enzyme activity could be extracted from dense smooth muscle cell layers under the conditions described above. It should be mentioned that the use of Tris buffer, used in previous studies (Tuderman et al., 1978; Nusgens et al., 1980), resulted in rapid (90% in 14 days) inactivation of the enzyme. Using 0.1 M sodium cacodylate buffer, we demonstrated that 50% of the activity remained after 30 days of storage at 4 °C in the case of enzyme fraction I. The culture medium contained no active enzyme even after concentration using 60% ammonium sulfate precipitation.

The K_m value of the enzyme activity was measured by using six different substrate (pN-collagen type III) and two different enzyme concentrations (see Materials and Methods). The K_m value of the enzyme was determined to be 0.76 μM. A neutral pH was required for enzyme activity, the pH optimum being 7.4–7.6. No activity was seen at pH 5.5 or 9.0.

Calcium is said to be essential for the cleavage of N-propeptide from type III collagen (Nusgens, 1980). In our system, incubation buffer containing 2 mM $CaCl_2$ resulted in maximal enzyme activity, but 8 mM $CaCl_2$ inhibited the activity by up to 70% (see Table II). If $CaCl_2$ was left out of the assay buffer, the activity decreased to only 35% of that found in the

normal assay. Cu^{2+} and Zn^{2+} obviously inhibited the enzyme reaction as well as Mg^{2+} and Mn^{2+} (for more details, see Table II). Surprisingly, EDTA did not at first appear to have any effect on the enzyme activity at concentrations of 10–100 mM, but sufficient preincubation with the enzyme (>1 h) resulted in 70% inhibition even at a concentration of 2 mM. EGTA, a proteinase inhibitor that specifically binds calcium ions, also inhibited the enzyme when preincubated with it for 1 h, but not as efficiently as EDTA; 10 mM NEM or 2 mM α,α'-dipyridyl did not have any effect on the enzyme. DTT was a potent inhibitor of enzyme activity: even at a concentration of 20 μM, total loss of enzyme activity was demonstrated. The serine protease inhibitor PMSF did not affect enzyme activity at concentrations of 1–100 μM.

Fetal calf serum did not inhibit the enzyme activity, and there was even a small increase of activity at serum concentrations varying from 4 to 10%. Lysine, a suggested inhibitor of carboxy-terminal protease (Morris et al., 1979; Ryhänen et al., 1982), also did not affect the enzyme activity under the present conditions (see Table II).

Discussion

In this study, we have partially purified an enzyme cleaving the amino-terminal propeptide from type III pN-collagen by using cultured smooth muscle cells as the starting material. These cells in dense cultures produce type III collagen at up to 50% (Mayne et al., 1978; Beldekas et al., 1981) of their total collagen production, thus being a good source of enzymes processing type III procollagen. We did not find any enzyme activity in the cell medium, in contrast to previously reported results in which type III N-terminal endopeptidase was purified from calf tendon fibroblast culture medium (Nusgens et al., 1980). This may reflect differences in enzymes from different species, tissues, or cell types. It should also be noted that no data concerning the ratio of cell layer to medium enzyme activity were given in the previous study (Nusgens et al., 1980), and thus the medium enzyme reported might represent only a small fraction of the total activity. The purification procedure described here requires the use of only two affinity columns and results in a relatively good purification coefficient and a high yield of specific activity.

The amino-terminal protease activity purified here seems to have a molecular weight of close to 72 000, but we also found activity in fraction II containing an additional major component with a molecular weight of 145 000. It is possible that the latter is another, possibly dimeric form of the enzyme with at least partially similar enzyme activity. It cannot be excluded, however, that the M_r 145 000 molecule is a contaminant, closely attached to the enzyme molecule, and the activity is caused by the M_r 72 000 component, constantly present in fraction II. The larger molecule obviously covers the part of the enzyme molecule having affinity for concanavalin A since the enzyme activity in fraction II did not attach to this matrix, in contrast to the enzyme in fraction I, with a molecular weight of 72 000.

The specificity with which the amino-terminal protease purified here cleaves type III procollagen was not established definitively. However, the results suggest that the cleavage site of this neutral proteolysis is close to that occurring in vivo. The larger reaction products comigrated with type III collagen α chains, and smaller products have a molecular weight close to that of purified type III N-propeptide (Engel et al., 1977). The specificity of the purified enzyme for type III procollagen was also supported by the observation that denatured pN III collagen was not cleaved in significant quantities, this finding being analogous to those reported concerning an amino-terminal protease specific for type I procollagen (Tuderman et al., 1978; Tuderman & Prockop, 1982). It appears that correct conformation of the cleavage site is critical for the action of both procollagen amino-terminal proteases described so far.

Our findings confirm the previous suggestions based on studies with enzymes from chicken tendon fibroblasts and cultured calf tendon fibroblasts that types I and III collagen amino-terminal proteases are different proteins (Tuderman et al., 1978; Nusgens & Lapiere, 1979; Nusgens et al., 1980; Tuderman & Prockop, 1982). We demonstrated no cleavage of type I procollagen or type IV procollagen with our partially purified enzyme. The amino-terminal protease of type I procollagen purified and characterized by Tuderman et al. (1978, 1982) did not cleave type III or IV procollagen, and Nusgens et al. (1980) were also unable to demonstrate cleavage of type I procollagen by using neutral proteinase from calf tendon fibroblast medium. Also, other characteristics like metal cofactor requirements and response to various protease inhibitors of the enzymes purified here and to any extent previously (Tuderman et al., 1978, 1982; Nusgens et al., 1979, 1980; Leung et al., 1979; Morris et al., 1979) justify the conclusion that type I and type III procollagens are processed by different N-terminal enzyme proteases. Contrary to this, the C-terminal protease may be the same protein for all the procollagens of the extracellular matrix (Njieha et al., 1982).

How the two fractions of enzyme activity reported here are related to each other and to previously reported type III amino-terminal endopeptidase activities with molecular weights of 150 000 and 100 000 (Nusgens, 1980) remains an open question. The immunological characterization of two fractions of our enzyme activity is under process.

Acknowledgments

We thank Dr. Karl Tryggvason for donating type IV procollagen and for critical reading of the manuscript and Dr. Heikki Seppä for providing the first batches of smooth muscle cells for our experiments. We gratefully acknowledge the advice and valuable comments given at all stages of this study by Professor Kari I. Kivirikko. We also acknowledge the expert technical assistance of Eeva Makkonen.

Registry No. Procollagen N-terminal proteinase, 68651-94-5.

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Different Effects of Substitution of the Near-Invariant Glutamine-4 on the Properties of Porcine and Bovine Pancreatic Phospholipases A_2†

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ABSTRACT: The precise role of the near-invariant Gln-4 residue in bovine and porcine pancreatic phospholipases A_2 was investigated with semisynthesis. Both in bovine and in porcine ϵ-amidinated phospholipases A_2, Gln-4 was substituted by Glu, Asn, and Nle. Binding and kinetic experiments revealed that replacement of Gln-4 by Asn or Nle in bovine phospholipase A_2 eliminates most of the activity, whereas a Gln-4 → Glu substitution affects the enzymatic activity but not the affinity for neutral aggregated substrates. These results clearly indicate the absolute requirement of an $O^{\epsilon 1}$ function of Gln or Glu at the 4-position in *bovine* phospholipase A_2 for a functional lipid binding domain. In contrast, all the *porcine* phospholipase A_2 "mutants" show affinities for micellar aggregates comparable to that of the native enzyme and possess almost full catalytic activity in the kinetic assays with micellar and monomeric short-chain lecithins. The opposite effect of the substitution of Gln-4 by Nle or Asn on the properties of the two enzymes is most likely a result of the presence of a Glu residue at position 71 in the porcine enzyme instead of an Asn as in the bovine enzyme. The porcine phospholipase A_2 is known to possess a second Ca^{2+} binding site located at Glu-71 and affecting the N-terminal region. Recent X-ray data of bovine and porcine phospholipases A_2, showing different conformations of the peptide loop 59–71 in these two enzymes, are in good agreement with this explanation.

Phospholipase A_2 (EC 3.1.1.4) specifically catalyzes the hydrolysis of all naturally occurring phospholipids in the presence of Ca^{2+}. The enzyme is found in low concentrations in almost every cell or cellular particle studied (van den Bosch, 1980) but is most abundant in snake venoms and mammalian pancreas (Slotboom et al., 1982). In the pancreas, the enzyme is synthesized as a zymogen. Upon secretion into the gastrointestinal tract, the proenzyme is converted into the active enzyme by limited tryptic proteolysis (de Haas et al., 1968). This active enzyme possesses, in addition to the low activity on monomeric zwitterionic substrates, a 10^3–10^4-fold higher catalytic activity on organized lipid–water interfaces such as occur in micelles (Volwerk & de Haas, 1982).

Modification and substitution of the N-terminal Ala-1 in the porcine pancreatic enzyme revealed that the presence of a free α-NH_3^+ group on Ala-1 is essential for binding to

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0006-2960/84/0423-1256$01.50/0