Partial Purification of a Procollagen C-Proteinase. Inhibition by Synthetic Peptides and Sequential Cleavage of Type I Procollagen†

Francois K. Njieha, Tadanori Morikawa,‡ Leena Tuderman,§ and Darwin J. Prockop*

ABSTRACT: A procollagen C-proteinase which cleaves the C-propeptides from type I procollagen was purified about 125-fold from membranous bones of chick embryos. As estimated by gel filtration, the enzyme was about 80000 daltons. When a reaction with modified procollagen was carried out, the enzyme preferentially cleaved the C-propeptides from the proα chains in the order proα1, proα1, and then proα2. The enzyme was inhibited by several metal chelators and high concentrations of dithiothreitol. It was also inhibited by 5% fetal calf serum. A series of inhibitors of serine proteinases and sulfhydryl-containing proteinases were not inhibitory. Four oligopeptides were synthesized with amino acid sequences similar to the amino acid sequences around the sites at which the C-propeptides are cleaved during the conversion of procollagen to collagen in vivo. The peptide Tyr-Tyr-Arg-Ala-Asp-Asp-Ala inhibited the enzyme 35–60% in concentrations of 6–12 mM. Shorter peptides containing the Ala–Asp bond cleaved by the enzyme were less effective. The partially purified enzyme was also found to cleave the C-propeptides from type II and type III procollagens.

At least two different neutral proteinases are required to convert interstitial procollagens to collagens. A type I procollagen N-proteinase removes the three N-propeptides from the N-terminal ends of the proα chains of type I procollagen. An enzyme with this activity was partially purified from calf tendon (Kohn et al., 1974), and subsequently similar enzymic activities were identified in several other tissues (Fessler et al., 1975; Tuderman et al., 1978; Leung et al., 1979; Morris et al., 1979; Steinmann et al., 1980; Nusgens & Lapière, 1979). Recently a type I N-proteinase was extensively purified from chick embryo tendons and characterized (Tuderman et al., 1978; Morikawa et al., 1980; L. Tuderman and D. J. Prockop, unpublished results). Also, an N-proteinase for type III procollagen was extensively purified from cultures of calf tendon fibroblasts (Nusgens et al., 1980). A procollagen C-proteinase which removes the three propeptides from the C-terminal end of type I procollagen has also been identified in a number of tissues and cell systems, including cultured human and mouse fibroblasts (Goldbert et al., 1975; Kessler & Goldberg, 1978), membranous bone from chick embryos (Duskin et al., 1978; Morris et al., 1979), and cultured tendon fibroblasts from chick embryos (Leung et al., 1979). The neutral procollagen C-proteinase found in cultured fibroblasts from chick embryo tendons was separated from the N-proteinase and partially characterized (Leung et al., 1979). An acidic proteinase which removes the C-terminal propeptides from type I procollagen has also been identified (Davidson et al., 1979).

We here report partial purification and characterization of a procollagen C-proteinase from chick embryo calvaria.

Materials and Methods

Materials. Pepsin-digested collagen from calf skin was a gift from Dr. Peter Bruckner. It was partially purified as described by Cawston & Barrett (1979). Other materials were obtained from sources previously specified (Tuderman et al., 1978).

Preparation of Procollagen Substrates. Type I procollagen substrates were prepared by using matrix-free cells from chick embryo tendons as described previously (Dehm & Prockop, 1972; Tuderman et al., 1978), but conditions were altered so that pCcollagen[1] was obtained in addition to procollagen. The cells were incubated at a concentration of 1.5×10^7/mL with a ^{14}C-labeled mixture of amino acids (New England Nuclear) for 6 h instead of the 4–5 h used previously. Also, the ammonium sulfate precipitate of the medium was chromatographed on a DEAE-cellulose (DE-52; Whatman) column at pH 8.6 instead of 7.4 (Hoffmann et al., 1976). Under these conditions, about 20% of the nondialyzable ^{14}C-labeled protein was recovered in the void volume of the column as collagen. About 25% eluted in a peak just after the void volume and consisted of pCcollagen. The remainder eluted as a peak in the latter two-thirds of the gradient and consisted of procollagen (Hoffmann et al., 1976). The fractions containing pCcollagen and procollagen were pooled separately and concentrated by precipitation with 176 mg/mL of ammonium sulfate for 4–5 h at 4 °C. After centrifugation, the samples were stored at −20 °C in 0.1 M Tris-HCl buffer, pH 7.5 at 25 °C, containing 0.4 M NaCl. The protein concentration was about 400 μg/mL.

Type II procollagen was prepared with matrix-free cells from chick embryo sternal cartilages (Dehm & Prockop, 1972). The cells were incubated at a concentration of 5×10^6/mL with 1 μCi/mL ^{14}C-labeled mixture of amino acids for 3 h in modified Krebs medium without fetal serum. The medium was precipitated with 176 mg/mL ammonium sulfate

† From the Department of Biochemistry, College of Medicine and Dentistry of New Jersey—Rutgers Medical School, Piscataway, New Jersey 08854. *Received April 24, 1981; revised manuscript received October 2, 1981.* This work was supported in part by National Institutes of Health Grant AM 16516.

‡ Present address: The Protein Research Foundation, Osaka, Japan.

§ Present address: Department of Structural Biology, Biocenter of the University of Basel, Basel, Switzerland.

[1] Abbreviations: pNcollagen, modified procollagen containing the N-terminal propeptides only; pCcollagen, modified procollagen containing the C-terminal propeptides only; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; NaDodSO$_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; PAB, *p*-aminobenzamidine; NEM, *N*-ethylmaleimide; PCMB, *p*-(chloromercuri)benzoate; DTT, dithiothreitol; B, benzyl; BCl, 2,6-dichlorobenzyl; Tos, *p*-toluenesulfonyl; Su, *N*-hydroxysuccinimide; HOBt, 1-hydroxybenzotriazole; DCC, dicyclohexylcarbodiimide; D–b, dicyclohexylcarbodiimide and 1-hydroxybenzotriazole; TLC, thin-layer chromatography; Boc, *tert*-butyloxycarbonyl; Aoc or *tert*-Aoc, *tert*-amyloxycarbonyl.

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and dissolved in 0.4 M NaCl and 0.1 M Tris-HCl, pH 7.4 at 4 °C, and then chromatographed on a DEAE-cellulose column under the same conditions used to isolate type I procollagen and pCcollagen.

Radioactivity labeled type III procollagen was obtained from the medium of cultured human skin fibroblasts (Peltonen et al., 1980). The fibroblasts were labeled with 1 μCi/mL ^{14}C-labeled mixture of amino acids for 24 h, and the medium proteins were purified by chromatography on DEAE-cellulose.

Assays for Procollagen C-Proteinase. A rapid acid was developed here for assay of procollagen C-proteinase. The assay was carried out on a final volume of 100 μL which contained 3–5 μg (30 000–50 000 cpm) of labeled pCcollagen, 4–5 units of enzymatic activity (Table I), 5 mM $CaCl_2$, and 0.15 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4 at 35 °C. The reaction was carried out for 4 h at 35 °C. It was terminated by adding 100 μL of 0.25 M EDTA, 10 μL of a solution containing 0.2 mg/mL carrier collagen from calf skin, and 80 μL of absolute ethanol. The sample was allowed to precipitate for 30 min at 4 °C and then shaken on a mechanical shaker at 4 °C for an additional 30 min. The precipitate was removed by centrifuging the samples at 15000*g* for 40 min. A 50-μL aliquot of the supernatant was assayed for ^{14}C.

In most experiments, enzymic activity was also assayed more specifically by polyacrylamide gel electrophoresis. The enzymic reaction was performed as described above. The reaction was stopped by addition of 30 μL of 250 mM EDTA, 30 μL of concentrated sample buffer containing 100 mM Tris-HCl buffer, 20% $NaDodSO_4$, 8 M urea, 0.01% bromophenol blue (pH 6.8), and either 40 μL of 200 mM iodoacetamide for nonreduced gels or 40 μL of 10% DTT for reduced gels. The samples were heated in a boiling water bath for 5 min and separated by electrophoresis on 6% $NaDodSO_4$–polyacrylamide gels. Fluorograms were prepared under standard conditions (Bonner & Laskey, 1974; Laskey & Mills, 1975).

For quantitation of the enzyme activity from fluorograms of polyacrylamide gels, the fluorograms were scanned on a Joyce-Loebl microdensitometer, and the peaks traced on paper were cut and weighed. Total enzymic activity was calculated as the difference in C-proteinase-susceptible bonds between the control sample of substrate incubated without enzyme and the sample of substrate incubated with enzyme.

The total number of susceptible bonds was calculated from the weighed peak of uncleaved pCcollagen and the two peaks of intermediates in the conversion of pCcollagen to α chains according to

$$S = 3x_1y_1 + 2x_2y_2 + x_3y_3$$

where the coefficients for each term is the number of C-proteinase susceptible bonds in each peak, x is the weight of each peak, and y is a factor to correct the weight of each peak for any losses of α chains when the protein is denatured prior to electrophoresis. For the calculation, each α chain was assumed to be 100 000 daltons and each C-propeptide 33 000 daltons. Therefore, the value of y_1 was 1.0, the value for y_2 was 1.33, and the value of y_3 was 2, and the formula became

$$S = 3x_1 + 2.7x_2 + 2x_3$$

Because of variability in the amount of ^{14}C applied to different wells in the gel, the total number of susceptible bonds was also corrected by the factor z/z_i, where z was the sum of the weights of all the peaks in a lane containing substrate only and z_i was the sum of the weights of all the peaks in the sample lane.

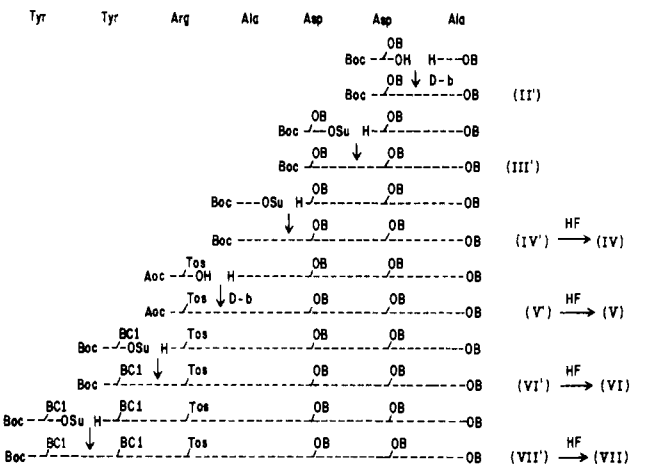

FIGURE 1: Scheme for the synthesis of peptides. For details, see Materials and Methods.

Purification of Procollagen C-Proteinase. For purification of the enzyme, calvaria were removed from 960 17-day-old chick embryos, washed in modified Kreb's medium, and weighed. The tissue, about 40 g, was frozen in liquid nitrogen and ground in a mortar and pestle. The powder obtained was extracted with 1.5 volumes of 250 mM Tris-HCl buffer (pH 7.5 at 25 °C) containing 750 mM NaCl, 25 mM $CaCl_2$, and 0.5% Triton X-100. The samples were extracted for 1 h at 4 °C with stirring and centrifuged for 30 min at 18000*g*. The residue was reextracted with 1 volume of the same buffer for 30 min at 4 °C, and the sample was recentrifuged. About two-thirds of the combined supernatants was chromatographed on a DEAE-cellulose column. Before chromatography, the sample was first dialyzed at 4 °C for 15 h against a medium salt buffer which consisted of 150 mM NaCl and 0.1% Triton X-100 in 50 mM Tris-HCl, adjusted to pH 7.5 at 25 °C. The sample was then dialyzed for 15 h against a low salt buffer which consisted of 50 mM NaCl and 0.2% Triton X-100 in 50 mM Tris-HCl, pH 7.5. The large precipitations which formed during the two dialysis steps were removed by centrifugation at 18000*g* for 30 min and discarded. The sample was then chromatographed in a 2.5 × 20 cm column of DEAE-cellulose (Whatman DE-52) in the low salt buffer. The column fractions were assayed with the rapid assay for C-proteinase, and the fractions containing enzymic activity were concentrated on an Amicon PM-30 filter. After a 10-fold concentration had been achieved, the sample was dialyzed against 50 mM Tris-HCl buffer (pH 7.5 at 25 °C) containing 150 mM NaCl and 5 mM $CaCl_2$. A small precipitate was removed by centrifugation at 18000*g* for 30 min, and then the sample was chromatographed on a 2.5 × 90 cm column of Sephadex G-150 (Pharmacia). The column fractions were assayed, and the peak containing enzymic activity was concentrated 10–15-fold on an Amicon PM-30 filter and stored at −20 °C.

General Procedures for Synthesis of Peptides. Peptides were prepared by stepwise elongation method in solution using Ala-OB as the starting material (Figure 1). All α-amino groups were protected with a Boc group except for Arg, where an Aoc group was used. The carbonyl group of Ala and β-carbonyl group of Asp were protected with B groups. Functional groups in side chains of Tyr and Arg were protected with BCl (Erickson & Merrifield, 1973) and Tos groups, respectively. Coupling reactions were carried out by the DCC-HOBt method (König & Geiger, 1970) for the preparation of compound II′ and V′ (Figure 1). Compounds III′, IV′, VI′, and VII′ were prepared by the active ester method

using OSu esters (Anderson et al., 1963).

After each coupling reaction, the product was purified by recrystallization or reprecipitation until sufficiently homogeneous material was obtained. Homogeneity was judged by TLC on silica gel. Protecting groups of fully protected linear peptides were removed simultaneously by a single treatment with HF at 0 °C for 20–40 min in the presence of anisole (Sakakibara & Shimonishi, 1965; Sakakibara et al., 1967). The crude products were purified by gel filtration on Bio-Gel P-2 (Bio-Rad) after treatment with Dowex 1-X4 (AcO^- form).

Synthesis of Peptide IV. III′ was homogeneous by TLC in chloroform–methanol–acetic acid (95:5:3). III′ (2.7 g, 3.9 mmol) was reacted with 10 mL of CF_3COOH at room temperature for 20 min. CF_3COOH was removed by evaporation, and the residue was dissolved in 5 mL of DMF and neutralized with triethylamine in an ice bath. Boc-Ala-OSu (1.3 g, 4.5 mmol) was added, and the solution was stirred at room temperature for 2 days. The reaction mixture was diluted with 150 mL of ethyl acetate and was washed with 0.5 HCl, 5% $NaHCO_3$, and water. The organic phase was dried over anhydrous $MgSO_4$ and was concentrated to yield 2.7 g of product IV′, from which 2.2 g was recrystallized with ethyl acetate and hexane. A single spot was obtained by TLC in chloroform–methanol–acetic acid (95:5:3). The fully protected tetrapeptide IV′ (98 mg, 0.128 mmol) was treated with approximately 5 mL of anhydrous HF at 0 °C for 40 min in the presence of 0.5 mL of anisole. Excess HF was then removed under reduced pressure at −5–0 °C. The residue was dissolved in 10 mL of 10% acetic acid, and the solution was extracted with 10 mL of ether. The aqueous phase was passed through a column of Dowex 1-X4 (AcO^-). The column was eluted with 50 mL of water, and the eluant was lyophilized to yield 52 mg of product. The crude material was dissolved in 0.05 M acetic acid and chromatographed on a 1.5 × 6.1 cm column of Bio-Gel P-2 (Bio-Rad), which was eluted with 0.5 M acetic acid. Fractions of 2.2 mL were collected. The absorbance of the fractions was assayed at 230 nm, and the major peak (fractions 34–38) was pooled and lyophilized to yield 37 mg of product IV. The product was homogeneous as tested by paper electrophoresis in 0.2 M pyridineacetate (pH 4.8) and by TLC in 1-butanol–acetic acid–water (4:1:1).

Synthesis of Peptide V. IV′ (2.0 g, 2.6 mmol) was treated with 7 mL of CF_3COOH for 20 min and was evaporated in vacuo. The residue was dissolved in 5 mL of tetrahydrofuran and neutralized with triethylamine. Aoc-Arg(Tos)-OH (1.23 g, 2.8 mmol) was added to the solution followed by addition of HOBt (350 mg, 2.6 mmol). The solution was cooled to −10 °C, and DCC (577 mg, 2.8 mmol) in 3 mL of tetrahydrofuran was slowly added. The solution was stirred at −10 °C for 3 h and then at 4 °C overnight. The precipitate was removed by filtration, and the filtrate was concentrated by evaporation. The residue was dissolved in 200 mL of ethyl acetate and was washed with 1 N HCl, 5% $NaHCO_3$, and water. The organic phase was dried over anhydrous $MgSO_4$ and was concentrated. The residue was not homogeneous as judged by TLC and was purified by column chromatography on silica gel using chloroform–ethyl acetate (1:1) and ethyl acetate–methanol (1:1) for elution. The eluate was checked by TLC, and the major peak was pooled and was evaporated to obtain 2.3 g (80.7%) of V′. A single spot was obtained by TLC in chloroform–methanol–acetic acid (95:5:3 and 80:10:5) and ethyl acetate–methanol (1:1). The protected pentapeptide V′ (126 mg, 116 μmol) was treated with HF and isolated by the same procedure described for the preparation of IV. The peptide was then purified on a 2.6 × 81 cm column of Bio-Gel P-2 which was eluted with 1 M acetic acid. Fractions of 4.0 mL were collected, and the absorbance of the fractions was measured at 230 nm after dilution with water. The major peak (fractions 74–80) was pooled and lyophilized to yield 48 mg of product V. The product was homogeneous as tested by paper electrophoresis in 0.2 M pyridineacetate (pH 4.8), and by TLC in 1-butanol–acetic acid–water (4:1:1) and in 1-butanol–acetic acid–pyridine–water (15:3:10:12).

Synthesis of Peptide VII. VI′ (1.3 g, 0.93 mmol) was treated with 5 mL of CF_3COOH for 30 min. CF_3COOH was removed by evaporation, and 50 mL of ether was added to yield a precipitate which was dried over NaOH in vacuo. This product was dissolved in 7 mL of DMF and neutralizing with triethylamine. Boc-Tyr(BCl)-OSu (552 mg, 1.02 mmol) was added, and the solution was stirred at room temperature for 3 days. The reaction mixture was slowly added to 100 mL of 0.5 N HCl in an ice bath. The precipitate that formed was isolated by filtration, washed with water, and dissolved in chloroform. The organic phase was extensively washed with 1 N HCl, water, 10% Na_2CO_3, and water and was dried over anhydrous $MgSO_4$. After evaporation, the residue was reprecipated from ethanol, ethyl acetate, and ether to yield 1.4 g of product VII′. A single spot was obtained by TLC in chloroform–methanol–acetic acid (95:5:3 and 80:10:5).

The fully protected heptapeptide VII′ (514 mg, 0.30 nmol) was treated with HF by the same procedure described for the preparation of IV. The crude material thus obtained was dissolved in a small amount of 1 M acetic acid and chromatographed on a 2.6 × 81 cm column of Bio-Gel P-2 (Bio-Rad), which was eluted with 1 M acetic acid. Fractions of 4.0 mL were collected. The absorbance of the fractions was measured at 280 nm, and the major peak (fractions 74–79) was pooled and lyophilized to yield 207 mg of product VII. The amino acid composition of peptide VII was the following: Tyr, 2.0; Arg, 1.1; Asp, 1.5; Ala, 1.8. Expected: Tyr, 2.0; Arg, 1.0; Asp, 2.0; Ala, 2.0.

Results

Rapid Assay for Procollagen C-Proteinase. So that the enzymic activity during purification could be assayed, a rapid assay for procollagen C-proteinase was developed. The assay was based in part on the rapid assays previously used for procollagen N-proteinase (Tuderman et al., 1978; Nusgens et al., 1980) and for procollagen C-proteinase (Kessler & Goldberg, 1978), in which the enzymic products were precipitated so that only the propeptides were recovered in the supernatant. So that the activity of C-proteinase in preparations which contained N-proteinase activity could be specifically assayed, we elected to employ ^{14}C-labeled pCcollagen as substrate and assay the release of the C-propeptide from this substrate. Uncleaved substrate, partially cleaved substrate, and native collagen, which was produced by cleavage of pC-collagen with the C-proteinase, were recovered in the precipitate (Figure 2). The C-propeptides were recovered in the supernatant of the rapid assay (Figure 2). The observed counts per minute in the assay increased with the amount of enzyme preparation added to the reaction system (Figure 3). The rapid assay was used for purification, but the specificity of all the preparations used here were verified by also assaying the reaction products by gel electrophoresis (Figure 2).

Partial Purification of Procollagen C-Proteinase. For extraction of C-proteinase activity, membranous bones (calvaria) of 17-day-old chick embryos were ground to a powder in liquid nitrogen. This technique appeared to provide higher yields of enzymic activity than either mincing or homogenizing the tissue.

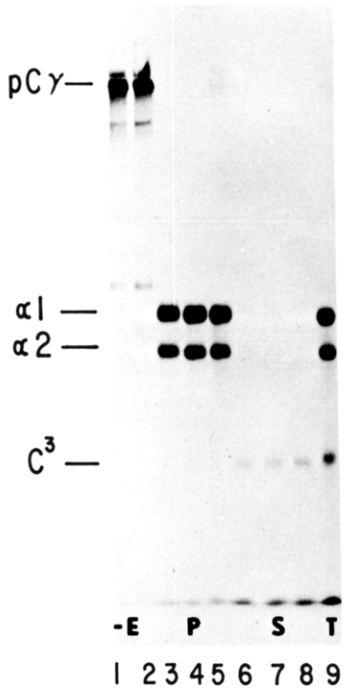

FIGURE 2: Analysis of C-proteinase reaction by polyacrylamide gel electrophoresis and fluorography. About 40 000 cpm of pCcollagen was incubated with 4 units of enzyme for 16 h. The reaction products were examined by electrophoresis on 6% gels without reduction of the samples. (Lanes 1 and 2) pCcollagen incubated without enzyme (-E); the major bands seen are pCγ chains. (Lanes 3–5) Precipitate obtained in rapid assay of reaction products (P); the two major bands seen are α1 and α2 chains. (Lanes 6–8) Supernatant obtained from rapid assay of reaction products (S); the major band is the disulfide-linked trimer of the C-propeptide (C^3). (Lane 9) Total products from enzymatic reaction (T).

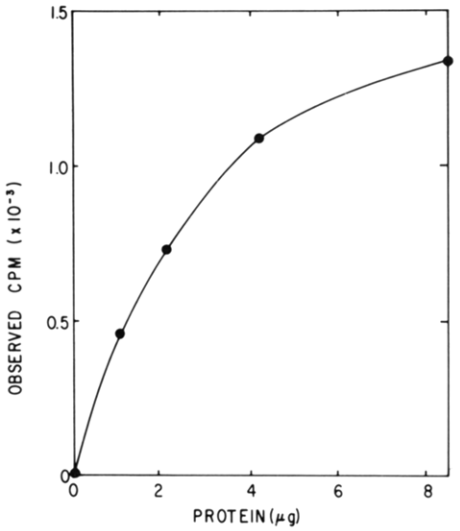

FIGURE 3: Rapid assay of C-proteinase. The enzymic reaction was carried out as described in text with partially purified enzyme (Table I).

Extraction of the activity with a high salt buffer containing 0.5% Triton X-100 gave better yields of activity than low salt or low detergent buffers. Dialysis of the initial extract against a medium salt buffer produced a heavy precipitate which did not contain enzymatic activity. Subsequent dialysis against the low salt buffer used for DEAE-cellulose chromatography gave an additional precipitate which also did not contain enzymic activity. Under the conditions employed here, the activity did not bind to DEAE-cellulose, but passing the preparation through a column of this material removed a large amount of nonenzyme protein. Also, some of the N-proteinase activity in the extract was bound to the column and could be eluted with a gradient of 0.2–1.0 M NaCl.

Table I: Purification of Procollagen C-Proteinase

enzyme fraction	total protein (mg)	total act. (units)[a]	sp act. (units/mg)	recovery (% of initial)
crude extract	2070	9320	4.5	100
DEAE column	548	9690	17.7	104
Sephadex G-150	4.69	2660	567	29

[a] One unit of C-proteinase activity is defined here as the amount of enzyme that releases 350 cpm of ^{14}C in the rapid assay described under Materials and Methods.

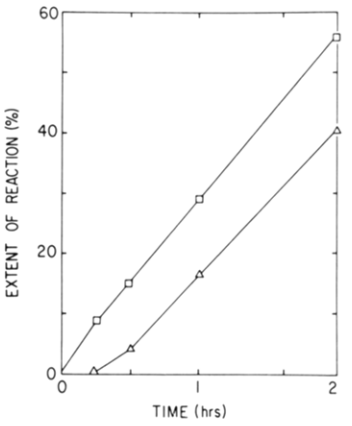

FIGURE 4: Release of α1 and α2 chains during enzymic cleavage of pCcollagen. About 40 000 cpm of pCcollagen were incubated with 6 units of partially purified C-proteinase. The reaction products were examined by gel electrophoresis, and fluorograms of the gels were evaluated by densitometry. The extent of the reaction was expressed as α1 chains or α2 chains released vs. total α1 chains or α2 chains expected with complete cleavage of the substrate (see text). Extent of reaction estimated by release of α1 chains (□); extent of reaction estimated by release of α2 chains (△).

A precipitate which did not contain enzymic activity was again encountered when the DEAE-column fractions were concentrated on an Amicon PM-30 membrane and dialyzed prior to gel filtration. As indicated in Table I, the overall purification was about 125-fold. The recovery of enzymic activity was about 30%. The final enzyme preparation employed here still contained procollagen N-proteinase activity. Since, however, the substrate used for all the enzymic reactions was pCcollagen, this activity did not interfere with any of the assays.

The enzymic activity eluted in about the middle of the total volume of the Sephadex G-150 column (not shown). Standardization of the column with globular proteins indicated that the apparent molecular weight was about 80 000.

Sequence in Which the pCα Chains in pCcollagen Are Cleaved by C-Proteinase. The substrate largely consisted of disulfide-linked pCcollagen but occasionally small amounts of lower molecular weight material was also seen (Figure 2, lanes 1 and 2). In preliminary experiments, it was found that when the reaction of enzyme with pCcollagen was carried out so that only part of the substrate was cleaved, α1 chains were released prior to and in greater proportion than α2 chains (Figure 4). This observation suggested that the C-properties of pCα1 chains were preferentially cleaved.

Sequential cleavage of the C-propeptides was also demonstrated in a second kinetic experiment. Here it was found that if the extent of reaction was expressed as release of the intact trimer of the three C-propeptides, there was an apparent time

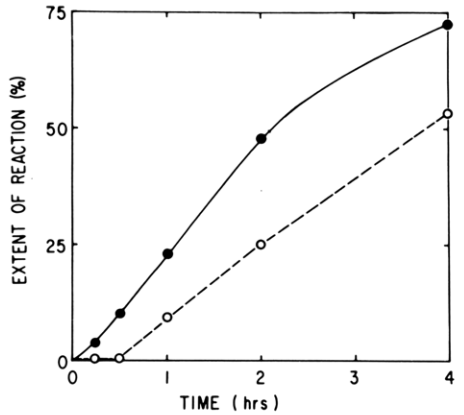

FIGURE 5: Release of C-propeptides during enzymic cleavage of pCcollagen. Experimental conditions as in Figure 4. Extent of reaction estimated by densitometry of pCγ and intermediate peaks with the formula described under Materials and Methods (●); extent of reaction expressed as release of C-propeptides in the same densitometric tracings (○).

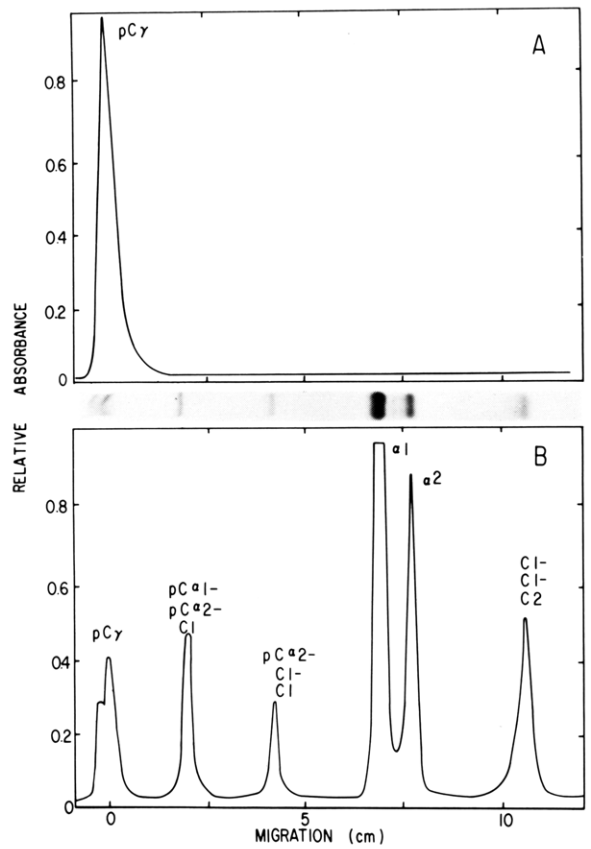

FIGURE 6: Analysis of pCcollagen partially cleaved with C-proteinase. About 40 000 cpm of pCcollagen was incubated with 4 units of enzyme for 2 h. The reaction products were examined by electrophoresis in a 6% polyacrylamide gel, and fluorograms of the gel were scanned with a densitometer. (A) pCcollagen incubated without enzyme. (B) pCcollagen incubated with enzyme. (Insert) Photograph of fluorogram used for densitometer tracing shown in (B). The band of pCγ appears split because it was recovered at the boundary between the stacking gel and the separating gel. As discussed in the text, the band of pCα1–pCα2–C1 may contain a small amount of pCα1–pCα1–C2. Also, the band of pCα2–C1–C1 may contain a small amount of pCα1–C1–C2.

lag in that essentially no C-propeptide was released in the first 30 min (broken line in Figure 5). However, there was no time lag if the data from the same experiment were expressed in terms of the number of peptide bonds cleaved with the formula presented under Materials and Methods.

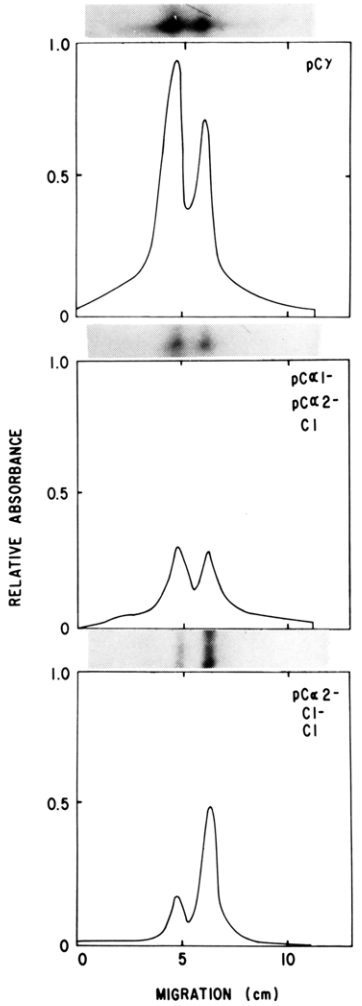

FIGURE 7: Second dimension of two-dimensional gel electrophoresis of intermediates in the cleavage of pCcollagen to collagen. Gel similar to that shown in Figure 6B was incubated with 10% DTT for 15 min at room temperature and placed horizontally on a second gel of 6% polyacrylamide for electrophoresis in the second dimension. (Top panel) Photograph and densitometer tracing of section of gel corresponding to first band (left to right) seen in Figure 6B. (Middle panel) Photograph and densitometer tracing of section of gel corresponding to second band seen in Figure 6B. (Lower panel) Photograph and densitometer tracing of section of gel corresponding to third band seen in Figure 6B. The direction of migration is left to right. The reduced C-propeptides appeared in the dye front and are not shown. In all three panels, the left-hand peak comigrates with pCα1 and the right-hand peak with pCα2.

For exploration of this observation further, a partial reaction was carried out, and the products were examined by two-dimensional gel electrophoresis in $NaDodSO_4$. The first dimension was electrophoresis without reduction (Figure 6). In the second dimension, the protein was reduced (Figure 7).

In the first dimension, six bands were seen (Figure 6B). As expected, the first band (left to right in Figure 6B), consisted of uncleaved pCcollagen and after reduction gave rise to pCα1 and pCα2 chains in a ratio of about 2:1 (upper panel in Figure 7). Also, as expected, the fourth and fifth bands in Figure 6B comigrated with α1 and α2 chains, respectively, in both dimensions. The sixth band comigrated with the trimer of the C-propeptides in the first dimension (Hoffmann et al., 1976) and after reduction gave rise to two bands which comigrated in 12% polyacrylamide gels with C1 and C2, the two C-propeptides from the proα1 and proα2 chains (not shown).

The second band (left to right in Figure 6B) gave rise, after reduction, to pCα1 and pCα2 chains in a ratio of about 1:1

Table II: Inhibition of Procollagen C-Proteinase

additions	concn (mM)	extent of inhibition (%) condition I[a]	condition II[a]
none		0	0
EDTA	2.5	70	70
	10	80	90
o-phenanthroline	1	85	90
α,α'-dipyridyl	5	60	10
DTT	2.5	100	100
PMSF	2	20	
	5	30	0
iodoacetamide	2	30	
	10	30	
NEM	10	20	0
PAB	10	10	5
Trasylol	100[b]	0	0
SBTI	0.02	0	0
β-aminopropionitrile	20	0	0
L-arginine	50	25	10
fetal calf serum	5[c]	100	

[a] Reaction conditions were varied so that either 53% (condition I) or 98% (condition II) of the susceptible bonds in the substrate was cleaved when no inhibitor was added. [b] Kallikrein inhibition units (FBA Pharmaceuticals). One unit is 0.14 μg of active material. [c] Value indicates the percent fetal calf serum in the reaction system.

(middle panel in Figure 7). It also gave rise to a C-propeptide which migrated at the dye front (not shown). Therefore, the second band in Figure 6B was identified as pCα1–pCα2–C1. Occasionally, when the products were examined in 4.5% polyacrylamide gels, a minor satellite band was seen with the second band. Therefore, the second band in Figure 6B probably contains a small amount of pCα1–pCα1–C2.

The third band in Figure 6B (left to right) gave rise, after reduction, primarily to pCα2 chains (lower panel in Figure 7) and C-propeptides (not shown). Therefore, this band was identified as pCα2–C1–C1. As indicated in Figure 7 (lower panel), a small amount of pCα1 was also present, and therefore a small amount of material in the third band was probably pCα1–C1–C2.

Because the major intermediates seen in the cleavage of pCcollagen to collagen were pCα1–pCα2–C1 and pCα2–C1–C1 (Figure 6B), the results indicated that the preferential order of cleavage was pCα1, pCα1, and then pCα2.

Inhibition by Proteinase Inhibitors. A series of proteinase inhibitors were tested with the partially purified C-proteinase. For detection of inhibitors which might prevent cleavage of the pCα1 chain but not the pCα2, the agents were tested under two conditions, one in which 53% of the susceptible bonds were cleaved in the control sample and a second in which 94% of the susceptible bonds were cleaved (Table II). The extent of inhibition was assayed by a densitometer tracing of fluorograms of slab gels and with the formula presented under Materials and Methods. As indicated in Table II, the enzymic reaction was inhibited by the three metal chelators, EDTA, *o*-phenanthroline, and α,α'-dipyridyl. The reducing agent DTT inhibited activity in a concentration of 2.5 mM. However, a series of inhibitors of serine proteinases and sulfhydryl-containing proteinases had a minimal effect on the enzyme. The agents in these categories which were tested included PMSF, iodoacetamide, NEM, PAB, Trasylol, and SBTI. The aldehyde reagent β-aminopropionitrile did not inhibit. Also, arginine, which was previously reported to inhibit C-proteinase (Leung et al., 1979), had a minimal effect. The enzyme, however, was completely inhibited by 5% fetal calf serum.

Table III: Inhibition of C-Proteinase with Synthetic Peptides

		concn (mM)	inhibition[a] (%)
IV	Ala-Asp-Asp-Ala	13	15
		26	30
V	Arg-Ala-Asp-Asp-Ala	9	20
		18	30
VI	Tyr-Arg-Ala-Asp-Asp-Ala	7	30
		14	45
VII	Tyr-Tyr-Arg-Ala-Asp-Asp-Ala	6	45
		12	60

[a] The synthetic peptides were incubated at 25 °C with 6 units of C-proteinase enzymic activity for 15 min before pCcollagen was added. The reaction was adjusted so that about 80% of the susceptible bonds were cleaved in the control sample. The reaction was assayed by densitometry of fluorograms of polyacrylamide gels.

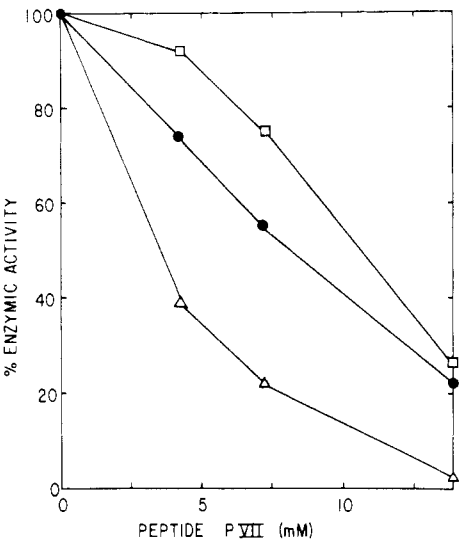

FIGURE 8: Inhibition of C-proteinase by peptide VII. Conditions as described in text. About 70% of the susceptible bonds in the pC-collagen was cleaved in the control sample. Enzymic activity expressed as percent of total susceptible bonds cleaved (●); enzymic activity expressed as percent of pCα1 chains cleaved (□); enzymic activity expressed as percent of pCα2 chains cleaved (△).

Inhibition of C-Proteinase with Synthetic Peptides. The peptide bonds in the chick proα1(I) and proα2 chains which are cleaved by procollagen C-proteinase have been identified by amino acid sequencing of the C-propeptide (Pesciotta, 1981) and by nucleotide sequencing of cloned cDNAs (Fuller & Boedtker, 1981; Dickson et al., 1981). The peptide bond cleaved in the proα1(I) chain is an Ala–Asp bond in the sequence Tyr-Tyr-Arg-Ala-Asp-Asp-Ala. In the proα2 chain, the bond cleaved is also an Ala–Asp bond in the similar sequence Tyr-Tyr-Arg-Ala-Asp-Gln-Pro. Four oligopeptides with amino acid sequences similar to the cleavage site in proα1(I) chain were synthesized (Figure 1 and Table III). All four of the peptides inhibited the cleavage of ^{14}C-labeled pC-collagen when added to the standard reaction system, and the effectiveness of the peptides as inhibitors increased with increasing length of the peptide. The longest peptide, peptide VII, inhibited the overall cleavage of susceptible bonds by 45–78% when present in concentrations of 6–12 mM (Figure 8 and Table III). Inhibition of cleavage of the pCα2 chain, however, was greater than the inhibition of the cleavage of the pCα1 chain. For example, with 7 mM peptide VII, cleavage

of the pCα2 chain was inhibited about 80%, but inhibition of the overall reaction was only about 45% and inhibition of cleavage of the proα1 chain was only about 25% (Figure 8).

Further experiments indicated the peptide VII was not an effective substrate for the reaction. The peptide at a concentration of 5–12 mg/mL was incubated with 6 units of partially purified C-proteinase for 6 h. The reactive products were assayed by TLC on silica-coated plates and with a solvent system of 1-butanol–acetic acid–water (4:1:1). One of the expected products, the tripeptide Asp-Asp-Ala, was well separated from peptide VII in the TLC system. Cleavage of 5% of the peptide VII would have been detectable under the conditions used. However, none of the expected product was detected (not shown).

In parallel experiments, peptide VII was tested as a potential inhibitor of procollagen N-proteinase. ^{14}C-Labeled procollagen was incubated with a highly purified preparation of procollagen N-proteinase (Tuderman et al., 1978; L. Tuderman and D. J. Prockop, unpublished results). The reaction system contained 50 000 cpm of ^{14}C-labeled procollagen and about 0.2 unit of N-proteinase. The reaction was carried out for 4 h at 30 °C, and the products were assayed electrophoretically. Densitometry of fluorograms of the gels indicated that in the control sample about 40% of the procollagen was converted to pCcollagen. The same value was obtained when 12 mM peptide VII was present in the reaction system, indicating the peptide did not inhibit the N-proteinase.

Cleavage of Type II and Type III Procollagens. The partially purified C-proteinase was found to cleave the C-propeptides from type II and type III procollagens.

As indicated in Figure 9 incubation with the enzyme converted proγ and pCγ chains in type II pCcollagen to α1(II) chains and a fragment with about the same mobility as the disulfide-linked trimer of C-propeptides from type I procollagen. pNα1(II) chains present in the substrate preparation were partially cleaved. Similarly, proα1(III) and pCα(III) chains in a preparation of type III procollagen were converted to α1(III) chains (Figure 9). A fragment with about the same mobility as the disulfide-linked trimer of the C-propeptides of type I procollagen was also seen when the same reaction products were examined by gel electrophoresis in $NaDodSO_4$ without reduction (not shown).

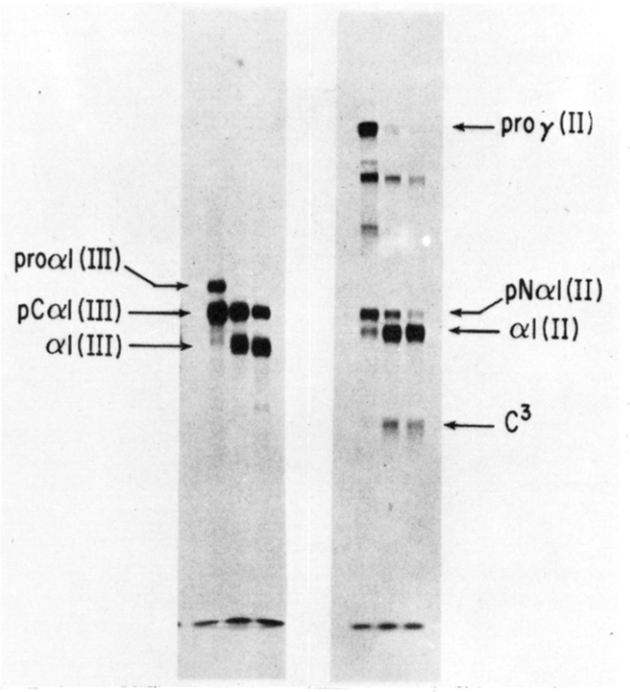

FIGURE 9: Cleavage of type II pCcollagen and type III procollagen. Type II pCcollagen was isolated from chick embryo cartilage cells, and type III procollagen was isolated from cultures of normal human skin fibroblasts as described in text. The substrates, about 35 000 cpm of each, were incubated with enzyme for 1.5 and 3 h. (Left panel) Type III procollagen after reduction. (Lane 1) Type III procollagen incubated without the enzyme. (Lanes 2 and 3) The same as in lane 1 incubated with 2.5 units of enzyme for 1.5 and 3 h, respectively. (Right panel) Type II procollagen not reduced. (Lane 1) Type II procollagen incubated without the enzyme. Some partially cleaved material is present. (Lanes 2 and 3) The same procollagen as in lane 1 incubated with 2.5 units of enzyme for 1.5 and 3 h, respectively.

Discussion

The development of a rapid assay for procollagen C-proteinase made it possible here to partially purify the enzyme. Partial purification, in turn, made it possible to extent characterization of this protein. By gel filtration, the C-proteinase appeared to be about 80 000 daltons. Inhibition of the enzyme with chelating agents confirmed previous indications that it is a metal-requiring proteinase. The studies with inhibitors also indicated that it was not a serine- or sulfhydryl-proteinase. The enzyme was, however, readily inhibited by serum, much as is observed with vertebrate collagenase (Harper, 1980) and procollagen type III N-proteinase (Nusgens et al., 1980).

Sequential cleavage of the C-propeptides from procollagen or pCcollagen was previously observed by two laboratories (Davidson et al., 1977; Morris et al., 1979). The results obtained here agree with the previous reports in demonstrating that intermediates in the conversion of pCcollagen to collagen can be readily recovered. The data here, however, indicate that the preferential order of cleavage is pCα1, pCα1, and then pCα2. The data of Davidson et al. (1977) and those of Morris et al. (1979) suggested that the first step was random cleavage of pCα1 or pCα2, the second was cleavage of pCα2, and the last was cleavage of pCα1. The reasons for the discrepancies are not apparent. Davidson et al. (1977) examined intermediates extracted from calvaria of chick embryos at a single time point. Morris et al. (1979) followed the kinetics of the reaction using procollagen proteinase from cultures of chick embryo fibroblasts and partially cleaved pCcollagen from calvaria of chick embryos as a substrate. These differences in experimental conditions may be critical.

The partially purified procollagen C-proteinase was inhibited by synthetic peptides with amino acid sequences similar to the amino acid sequence around the site in the proα1(I) chain which is cleaved in the conversion of procollagen to collagen in vivo. The same peptides did not inhibit procollagen N-proteinase. The results obtained with the synthetic peptides helped to establish the specificity of the procollagen C-proteinase studied here. The greater inhibition of cleavage of the pCα2 chain than that of the pCα1 chain may reflect the fact that the synthetic peptides had sequences identical with the pCα1 cleavage site but slightly different from the pCα2 cleavage site. It may also be a reflection of the sequential order in which the chains are cleaved. As was found with substrate analogues of procollagen N-proteinase (Morikawa et al., 1980), relatively high concentrations of the synthetic peptides were required to inhibit the C-proteinase. It may or may not be possible to develop more effective inhibitors of both the N- and C-proteinases by chemical modification of the peptides tested here and previously.

The observation that the partially purified C-proteinase also removed the C-propeptides from type II pCcollagen and type III procollagen suggests that the same enzyme acts on all three kinds of procollagen. However, the specificity of the activity for the cleavage of the type II and type III propeptides was

not examined in detail.

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References

Anderson, G. W., Zimmerman, J. E., & Callahan, F. (1963) *J. Am. Chem. Soc. 85*, 3039.
Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83.
Cawston, E. T., & Barrett, J. A. (1979) *Anal. Biochem. 99*, 240.
Davidson, J. M., McEneany, L. S., & Bornstein, P. (1977) *Eur. J. Biochem. 81*, 349.
Davidson, J. M., McEneany, L. S., & Bornstein, P. (1979) *Eur. J. Biochem. 100*, 551.
Dehm, P., & Prockop, D. J. (1972) *Biochim. Biophys. Acta 264*, 375.
Dickson, L. A., Ninomiya, Y., Bernard, M. P., Pesciotta, D. M., Vogeli, G., Pastan, I., Fietzek, P. P., Olsen, B. R., Parsons, J., Green, G., Eikenberry, E. F., & deCrombrugghe, B. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* (in press).
Duksin, D., Davidson, J. M., & Bornstein, P. (1978) *Arch. Biochem. Biophys. 185*, 3012.
Erickson, B. W., & Merrifield, R. B. (1973) *J. Am. Chem. Soc. 95*, 3750.
Fessler, L. H., Morris, N. P., & Fessler, J. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4905.
Fuller, F., & Boedtker, H. (1981) *Biochemistry 20*, 996.
Goldberg, B., Traubman, M. B., & Rabin, A. (1975) *Cell (Cambridge, Mass.) 4*, 45.
Harper, E. (1980) *Annu. Rev. Biochem. 49*, 1063.
Hoffmann H.-P., Olsen, B. R., Chen, H.-T., & Prockop, D. J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4304.
Kessler, E., & Goldberg, B. (1978) *Anal. Biochem. 86*, 463.
Kohn, L. D., Iserky, C., Zypnik, J., Lenaers, A., Lee, G., & Lapière, C. M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 40.
König, W., & Geiger, R. (1970) *Chem. Ber. 103*, 788.
Laskey, R. A., & Mills, A. D. (1975) *Eur. J. Biochem. 56*, 335.
Leung, M. K. K., Fessler, L. I., Greenberg, D. B., & Fessler, J. H. (1979) *J. Biol. Chem. 254*, 224.
Morikawa, T., Tuderman, L., & Prockop, D. J. (1980) *Biochemistry 19*, 2646.
Morris, N. P., Fessler, L. I., & Fessler, J. H. (1979) *J. Biol. Chem. 254*, 11024.
Nusgens, B. V., & Lapière, C. M. (1979) *Anal. Biochem. 95*, 406.
Nusgens, B. V., Goebels, Y., Shinkai, H., & Lapière, C. M. (1980) *Biochem. J. 191*, 699.
Peltonen, L., Palotie, A., Hayashi, T., & Prockop, D. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1621.
Pesciotta, D. M. (1981) Ph.D. Thesis, The State University of Rutgers, Piscataway, NJ.
Sakakibara, S., & Shimonishi, Y. (1965) *Bull. Chem. Soc. Jpn. 38*, 1412.
Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M., & Sugihara, H. (1967) *Bull. Chem. Soc. Jpn. 40*, 2164.
Steinmann, B., Tuderman, L., Peltonen, L., Martin, G. R., McKusick, V. A., & Prockop, D. J. (1980) *J. Biol. Chem. 255*, 8887.
Tuderman, L., Kivirikko, K. I., & Prockop, D. J. (1978) *Biochemistry 17*, 2948.

Association of Globin Ribonucleic Acid and Its Precursors with the Chicken Erythroblast Nuclear Matrix†

David A. Ross, Ray-Whay Yen, and Chi-Bom Chae*

ABSTRACT: Nuclear matrix was prepared from both erythroblasts and erythrocytes of chicken red blood cells. Greater than 90% of the globin nuclear RNA remains bound to the erythroblast nuclear matrix. There are approximately 1000 copies of globin RNA in the nucleus per cell, and most of these contain a poly(A) tail. Precursor β globin RNA exists in four high molecular weight forms, some of which are larger than the natural β globin gene. Most of the ribosomal RNA is lost during the preparation of an erythroblast nuclear matrix. In contrast, some of the snRNAs are specifically enriched in the erythroblast nuclear matrix. There is little or no globin nuclear RNA in the erythrocyte nuclear matrix. There appears to be no selective attachment of the globin genes to the erythroblast nuclear matrix. The nuclear matrix is postulated to be a platform for the differential processing of nuclear RNA.

An underlying structure of all eukaryotic nuclei is the nuclear matrix, nuclear lamina, or nuclear skeleton which morphologically resembles the nucleus but which is mainly composed of polymeric protein fibrils and RNA (Grebanier & Pogo, 1979). The nuclear matrix, obtained by digestion with DNase I or endogenous nucleases and repeated extraction with 2 M NaCl, contains a small amount of tightly bound and highly protected DNA and much of the nuclear RNA (Miller et al., 1978a,b; Berezney & Coffey, 1975). The nuclear lamina, the peripheral layer of the nuclear matrix, or nuclear skeleton can be prepared by slight variation of the digestion and washing steps used in preparing the nuclear matrix (Kaufman et al., 1981). Although it is not yet clear if the matrix has any nuclear function other than providing the structural framework of the cell nucleus, several pieces of evidence suggest that the nuclear matrix may serve as a platform for various important nuclear events. For example,

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