spheres of radius r and partial specific volume $\bar{v}$:

$$M = Nm = \frac{4\pi}{3}\frac{Nr^3}{\bar{v}}$$

$$I_1 = I_2 = I_3 = \frac{2}{3}I = \frac{8\pi}{15}\frac{r^5}{\bar{v}} \quad (8)$$

which, using eq 6 and 7 leads to:

$$G_r = -RT \ln[\beta(RT)^{3/2}M^{5/2}] \quad (9)$$

$$\beta = 75.1\bar{v}/N^4h^3 \quad (10)$$

For nonspherical or inhomogeneous particles, the molecular weight M may be replaced in eq 9 by M', the molecular weight of spherical particles having the same value as the $I_1I_2I_3$ product.

Then, the translational and rotational contributions to the free energy of a dissociation of the complex AB are simply:

$$\Delta G_t = G_t^A + G_t^B - G_t^{AB}$$

$$= -RT \ln\left[\alpha(RT)^{3/2}\left(\frac{M_AM_B}{M_A + M_B}\right)^{3/2}\right] \quad (11)$$

$$\Delta G_r = G_r^A + G_r^B - G_r^{AB}$$

$$= -RT \ln\left[\beta(RT)^{3/2}\left(\frac{M'_AM'_B}{M'_{AB}}\right)^{5/2}\right] \quad (12)$$

The free energy released is that of a particle of molecular weight $M_AM_B/(M_A + M_B)$, the moment of inertia of which corresponds to a sphere of molecular weight $M'_AM'_B/M'_{AB}$. The latter depends somewhat on the geometry of the complex. In the case where one of the molecules is much heavier than the other, eq 11 and 12 show that the free energy released is essentially that of the small molecule.

The above theory assumes that the molecules move and rotate in the absence of external forces; in other words, that they behave like perfect gases. Is that approximation reasonable for aqueous solutions? Interactions between solute molecules may be neglected, provided experimental data are extrapolated to infinite dilutions. Volume exclusion due to the solvent is easily taken into account in the calculation of G_t. Due to the loose packing of water, which leaves empty more than half of its total volume, this effect is small (about 0.4 kcal/mol). Much more important, interactions made with the solvent affect the partition functions Z_t and Z_r in a way which is difficult to estimate.

Still, the "perfect gas" theory has been shown to yield reasonable results in predicting rates of chemical reactions in solution (Page & Jencks, 1971), as it does for gases. Because the rates are very sensitive to the values of the free energies (of activation) involved, the agreement of calculated rates with experimental values indicates that the theory gives a correct estimate of the translational/rotational free energy (at least within a few kcal/mol).

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Partial Purification and Characterization of a Neutral Protease Which Cleaves the N-Terminal Propeptides from Procollagen†

Leena Tuderman, Kari I. Kivirikko,§ and Darwin J. Prockop*

ABSTRACT: A rapid assay procedure was developed for cleavage of the N-terminal propeptides of procollagen. With the assay a neutral procollagen N-protease was purified about 300-fold from chick embryo tendon extract. The enzyme had an apparent molecular weight of 260 000 and a pH optimum of 7.4. Ca^{2+} was required for enzymic activity but this requirement was partially replaced by Mg^{2+} or Mn^{2+}. The enzyme was bound to concanavalin A–agarose and therefore was presumably a glycoprotein. The N-propeptides released from type I procollagen were of about 23 000 and 11 000 daltons as estimated by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The partially purified enzyme was also found to cleave type II procollagen and the N-propeptide obtained was about 18 000 daltons. Heat denaturation of either type I or type II procollagen decreased the rate at which the proteins were cleaved by the N-protease.

The three proα chains of procollagen contain propeptide extensions at both their N- and C-terminal ends, and these propeptides must be removed before the molecule can form stable collagen fibers (for reviews, see Martin et al., 1975; Grant & Jackson, 1976; Prockop et al., 1976; Bornstein & Traub, 1978). Several observations have been made as to how the propeptides are removed and the enzymes involved. Genetic defects in the conversion of type I procollagen to collagen occur in cattle (Lapière et al., 1971; Lenaers et al., 1971), sheep (Fjølstad & Helle, 1974; Schofield & Prockop, 1973), and man (Lichtenstein et al., 1973), and these genetic defects are characterized by the presence of pNcollagen in which the C-propeptides are missing but the N-propeptide of the two proα1 chains and at least part of the N-propeptide of the proα2

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chain are retained. Tissue extracts from calves with the genetic defect did not cleave pNcollagen whereas extracts of normal calf tissues cleaved the protein (Lapière et al., 1971). The defect in calves appeared therefore to be a deficiency of a procollagen N-protease[1] and an enzyme with N-protease activity was partially purified from calf tendon (Kohn et al., 1974). An apparently specific procollagen C-protease activity was detected in the media of fibroblast cultures and experiments with tunicamycin suggested that the C-protease was a glycoprotein (Duksin & Bornstein, 1977). Further evidence for the presence of separate N- and C-proteases was provided by observations indicating that both pNcollagens and pCcollagens can be recovered from organ and cell cultures (Bornstein et al., 1972; Goldberg et al., 1972, 1975; Uitto et al., 1972; Goldberg & Scherr, 1973; Layman & Ross, 1973; Fessler et al., 1975; Davidson et al., 1977; Uitto, 1977).

In the present report we describe partial purification and characterization of a neutral protease from chick embryo tendon extract which removes the N-propeptides from both type I and II procollagens.

Materials and Methods

Preparation of Procollagen Substrate. Radioactively labeled type I procollagen was prepared as described previously. Cells were isolated by collagenase and trypsin digestion of tendons from 17-day old chick embryos (Dehm & Prockop, 1972), and about 2.0 to 2.5 × 10^9 cells were incubated at a concentration of 7.5 × 10^6 cells/mL with 200 μCi of [^{14}C]-proline or [^{35}S]cystine (New England Nuclear Corp.) for 4 h at 37 °C in modified Krebs medium (Dehm & Prockop, 1972) without fetal calf serum. The medium was treated with protease inhibitors and precipitated with 176 mg/mL of ammonium sulfate prior to chromatography on DEAE-cellulose (Smith et al., 1972; Hoffmann et al., 1976). The fractions containing the radioactive procollagen were pooled and dialyzed against 0.4 M NaCl in 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C. The sample was precipitated with 176 mg/mL ammonium sulfate and dissolved in about 3 mL of 0.4 M NaCl and 0.1 M Tris-HCl buffer. Assays of hydroxyproline (Kivirikko et al., 1967) indicated that the final concentration of procollagen was about 300 μg/mL. When [^{14}C]proline was used as the radioactive label, the specific activity was 10 000 to 20 000 cpm/μg; when [^{35}S]cystine was used, the specific activity was 3000 to 6000 cpm/μg. The procollagen was frozen in aliquots of about 0.2 mL which were thawed just prior to use. The radioactively labeled procollagen was homogeneous as tested by polyacrylamide gel electrophoresis (see below).

Radioactively labeled type II procollagen was prepared with cells obtained by enzymic digestion of sternal cartilages from 17-day old chick embryos (Dehm & Prockop, 1973). About 8 × 10^8 cells were incubated in a concentration of 5 × 10^6 cells/mL with 60 μCi of [^{14}C]proline for 4 h at 37 °C in modified Krebs medium without fetal calf serum. The medium was treated with protease inhibitors and precipitated with 176 mg/mL of ammonium sulfate. The precipitate was dissolved in 5 mL of 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C. The ammonium sulfate precipitation was repeated and the sample was dissolved in about 1 mL of 0.4 M NaCl and 0.1 M Tris-HCl buffer. The radioactively labeled protein consisted of proα1(II) when examined by polyacrylamide gel electrophoresis in $NaDodSO_4$ (see below).

Rapid Assay of Enzymic Activity. The assay was carried out in a final volume of 100 μL which contained 2–4 μg of labeled procollagen, 0.02 to 0.40 unit of enzyme, 5 mM $CaCl_2$, and 0.15 M NaCl in 0.05 M Tris-HCl buffer, pH 7.4, at 30 °C. The reaction was carried out for 90 min at 30 °C and it was stopped by transferring the tubes to an ice bath and immediately adding 50 μL of 500 mM EDTA, 100 μL of fetal calf serum heat inactivated at 60 °C for 15 min, and 750 μL of 243 mg/mL ammonium sulfate. The ammonium sulfate precipitation was allowed to proceed for 60 min at room temperature. The samples were then centrifuged at 15 000*g* for 30 min. A 0.5-mL aliquot of the supernate was placed in a scintillation vial, and 1.0 mL of water and 19 mL of Formula L-963 (New England Nuclear Corp.) were added for liquid scintillation counting. The background value for each assay was established with control samples containing all the components of the reaction mixture except enzyme. Background values varied from 60 to 120 cpm.

Purification of the N-Protease by Concanavalin A–Agarose Chromatography. Leg tendons were removed from about 150 17-day old chick embryos (about 10 g wet weight) and minced with scissors in 1 mL/g of cold 2 M KCl and 0.1% Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C. The samples were extracted for 2.5 h with occasional shaking. All these procedures were carried out at 4 °C. The sample was centrifuged at 15 000*g* for 30 min and the supernate was recovered. The pellet was resuspended in an equal volume of the extraction buffer, diluted 1:1 with water. The sample was centrifuged at 15 000*g* for 30 min and the supernate was combined with the first supernate. The extracts were stored at 4 °C for 1 to 4 days and extracts from two to four preparations were pooled. The pooled extracts were precipitated by adding an equal volume of 630 mg/mL ammonium sulfate in 0.1 M Tris-HCl, pH 7.4, at 4 °C. Then solid ammonium sulfate was added to give a final concentration of 390 mg/mL. The sample was stirred for 30 min and then centrifuged at 15 000*g* for 30 min. The precipitate was dissolved in 1 M KCl and 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C and dialyzed overnight against this same buffer.

A sample in volume of 45 mL or less (protein concentration of about 15 mg/mL) was passed through a 0.5–4.0 mL column of concanavalin A covalently linked to agarose (Con A–Sepharose; Pharmacia) which was equilibrated with 1 M KCl and 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C. The flow rate for the chromatography was 1 column volume/h. The column was washed with about 30 volumes of the equilibration buffer and then it was eluted with 15 column volumes of the same buffer containing 0.3 M α-methyl D-mannoside.

The fractions eluted with α-methyl D-mannoside were assayed for enzymic activity and the absorbance of the fractions was measured at 230 nm. The fractions having the highest specific activities were pooled and precipitated with 390 mg/mL ammonium sulfate as described above. The precipitate was dissolved in 1–2 mL of 1 M KCl and 0.1 M Tris-HCl, pH 7.4, at 4 °C and dialyzed overnight against this same buffer. The sample was then applied to a 1.5 × 90 cm mixed column of polyacrylamide and agarose (Ultrogel AcA 34, separation range 20 000 to 350 000 daltons; LKB). The column was equilibrated and eluted with 1 M KCl and 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C. Fractions of 2 mL were collected and fractions containing enzymic activity were pooled. The pooled fractions were concentrated in an ultrafiltration cell with a PM-10 membrane (Amicon).

[1] Abbreviations used: pCcollagen, collagen precursor which contains the C-terminal propeptides but not N-terminal propeptides; pNcollagen, collagen precursor which contains N-terminal propeptides but not C-terminal propeptides; N-protease, enzymic activity studied here which removes the N-terminal propeptides from procollagen; C-protease, enzymic activity which removes the C-terminal propeptides from procollagen; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetate; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Rapid Assay for Procollagen N-Protease Activity.

substrate[a]	treatment of substrate	radioact. in supernate: obsd values (cpm)	obsd values (% of total)	theor values[b] (% of total)
Expt 1				
[^{14}C]Pro-procollagen	none	100	0.8	0
[^{35}S]Cys-procollagen	none	120	1	0
[^{14}C]Pro-N-propeptide	none	1430	100	100
Expt 2				
[^{14}C]Pro-procollagen	α-chymotrypsin	1822	14	16
[^{35}S]Cys-procollagen	trypsin	3122	100	100
[^{14}C]Pro-procollagen	bacterial collagenase	10580	91	95
Expt 3				
[^{14}C]Pro-procollagen	N-protease	403	4[c]	
[^{35}S]Cys-procollagen	N-protease	1554	16[c]	

[a] The substrates used were procollagen which was obtained from tendon cells incubated with either [^{14}C]proline or [^{35}S]cystine and which was purified by DEAE-cellulose chromatography. The [^{14}C]proline-labeled N-propeptide was isolated from the medium of intact tendons (D. Pesciotta, M. Silkowitz, R. Berg, & B. Olsen, in preparation). [b] Theoretical values were calculated on the basis that 11% of the proline-labeled residues were in the N-propeptides (Becker et al., 1976; D. Hörlein & P. Fietzek, in preparation) and 5% in the C-propeptides (Olsen et al., 1977), and also on the basis that about half of the cystine-labeled residues were in the N-propeptides and about half in the C-propeptides. α-Chymotrypsin and trypsin were assumed to digest the propeptide domains to nonprecipitable fragments. Bacterial collagenase was assumed to digest all of the molecule except the C-propeptides to nonprecipitable fragments. [c] The extent of cleavage was 30% for both substrates if the data are adjusted to the assumption that the N-propeptides contain 11% of proline-labeled residues and 50% of the cystine-labeled residues found in procollagen.

Polyacrylamide Slab Gel Electrophoresis. Polyacrylamide slab gel electrophoresis was carried out as described by King & Laemmli (1971). Separating gels of 1.5-mm thickness were prepared from either 6% or 20% polyacrylamide and stacking gels from 6% polyacrylamide. The electrophoresis was carried out with about 50 mA for 4 h at room temperature.

The samples were prepared by dissolving ammonium sulfate precipitates of reaction mixtures in slab gel sample buffer consisting of 0.125 M Tris-HCl, pH 6.8, at room temperature, 2% $NaDodSO_4$ (Bio-Rad Laboratories), 10% glycerol, and 0.001% bromophenol blue (Eastman). The dissolved samples were heated at 100 °C for 3 min and then dialyzed against the sample buffer. When the whole reaction mixture was studied by slab gel electrophoresis, $1/10$ of the volume of 20% $NaDodSO_4$ was added to the mixture and it was then heated at 100 °C for 3 min. An equal volume of sample buffer was then added to the mixture and it was incubated overnight at room temperature. All samples were reduced by adding 5% (v/v) β-mercaptoethanol and by heating at 100 °C for 3 min just prior to electrophoresis.

The gels were stained by incubation for 1 h at room temperature with a solution containing 0.25% Coomassie Brilliant Blue R (Sigma Chemicals Co.) and 20% trichloroacetic acid, and they were destained in 7.5% acetic acid and 15% methanol. In experiments where radioactive bands were located in the gels, the gels were impregnated with 2,5-diphenyloxazole (PPO; Eastman) and dried under vacuum (Bonner & Laskey, 1974). They were then exposed to RP Royal "X-OMAT" x-ray films which had been "pre-flashed" to make their fluorographic response linear (Laskey & Mills, 1975).

Results

Assays for N-Protease Cleavage Products. A rapid procedure was developed here for purifying the enzyme and for observing the kinetics of the reaction. In most experiments, however, selected samples were also examined by electrophoresis on polyacrylamide gels to test the specificity of the enzymic cleavage.

The rapid assay was based on ammonium sulfate precipitation of native procollagen and of native pCcollagen. As reported previously, procollagen is readily precipitated with 30% saturated ammonium sulfate. In the assay developed here, however, fetal calf serum was used as a carrier to achieve more rapid and complete precipitation (Table I). The N-propeptide obtained by bacterial collagenase digestion of type I proα1 chains remained in the supernate (not shown). A peptide which was isolated from medium of intact chick tendons and which probably is the N-propeptide cleaved from the proα1 chain in situ was also recovered in the ammonium sulfate supernate (experiment 1, Table I). In contrast, the C-propeptide obtained after bacterial collagenase digestion of procollagen was largely precipitated. Therefore, the rapid assay developed here is not appropriate for assay of C-protease activity. Control experiments provided the expected results with [^{14}C]proline-labeled procollagen which had been digested with α-chymotrypsin or with [^{35}S]cystine-labeled procollagen which had been digested with trypsin (experiment 2, Table I). Procollagen which was heat denatured at 55 °C for 10 min was not precipitated. Therefore, it was essential that the procollagen substrate used for the reaction be in a native conformation.

Assay of N-Protease Activity in Extracts of Chick Embryo Tendons. Preliminary experiments demonstrated that an N-protease activity was recovered if minced tendons from 17-day old chick embryos were extracted with 1 M KCl and 0.1% Triton X-100 in 0.1 M Tris-HCl buffer, pH 7.4, at 4 °C. Lowering the KCl concentration to 0.15 M or increasing the concentration to 2.5 M decreased the yield of activity. Yields were not improved by increasing the concentration of Triton X-100 to 1% or by replacing this detergent with 0.1% or 1% Nonidet P-40 or 1% $NaDodSO_4$. Also, yields were not improved by adding either 30% ethylene glycol, 4 M urea, or 3 M sodium thiocyanate to the extraction buffer. Similarly, yields of activity were not improved by heating the minces in the extraction buffer at 55 °C for 4 min. Essentially the same amounts of activity were recovered when tendons from 16-, 17-, 18- or 19-day old chick embryos were employed but the specific activity of the extracts appeared to be somewhat higher when the 17-day old chick embryos were used.

The N-protease activity observed in extracts was proportional to the amount of extract added to the incubation mixture with 0.5–10 μL of tendon extract. Also, the reaction was linear for at least 90 min. The apparent K_m for the substrate was about 300 μg/mL or 1 μM procollagen but because of the limited availability of substrate, the value was not determined accurately.

The enzymic activity measured with the rapid assay apparently represented specific cleavage of the procollagen molecule, since examination of the reaction mixture on 6% polyacrylamide gels indicated a conversion of proα chains to

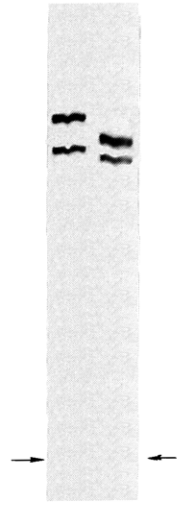

FIGURE 1: Polyacrylamide gel electrophoresis in $NaDodSO_4$ of reaction products with type I procollagen. Electrophoresis was carried out with 6% gel. About 2 μg of [^{14}C]proline-labeled procollagen incubated at 30 °C for 90 min in a reaction mixture not containing enzyme (slot 1), and with 0.4 unit of the enzyme (slot 2). The samples were reduced with 5% (v/v) β-mercaptoethanol. As indicated in the text, the new polypeptides in slot 2 comigrated with pCα1 and pCα2 chains.

TABLE II: Purification of Procollagen N-Protease from Chick Embryo Tendons.

enzyme fraction	total protein (mg)[a]	total act. (units)[b]	recovery (% of initial)	specific act (units/mg)
tendon extract	1152	1152	100	1.0
ammonium sulfate precipitate	664	1035	90	1.6
concanavalin A-agarose	14.1	808	70	57.3
gel filtration	1.75	534	46	306

[a] The protein concentration in tendon extracts was assayed by the ninhydrin assay of amino acids (Stein & Moore, 1948) after acid hydrolysis of the protein. Other samples were assayed by absorbance at 230 nm assuming $E_{230nm}^{1mg/mL}$ equal to 4.95. [b] Because of the variability in the specific activities of the radioactively labeled procollagen substrates, one unit of enzymic activity was temporarily defined as the amount of enzyme present in 1 mg of chick embryo tendon extract.

pCα chains (Figure 1). The migration position of the pCα1 and pCα2 chains was established by previous experiments in which it was shown that digestion of protein identified as pCcollagen with tadpole collagenase gave rise to peptides which comigrated with A fragments from the α1 and α2 chains of collagen and fragments which comigrated with the B fragments of procollagen (Olsen et al., 1976). As indicated below, the smaller fragments obtained after incubation of the procollagen with the tendon extracts were of the expected size of N-propeptides.

Purification of the N-Protease Activity. The N-protease activity found in the tendon extracts was removed when the extracts were incubated with concanavalin A linked to agarose prior to assay. Therefore a column of concanavalin A–agarose was used to purify the enzyme.

An extract from 35 g of minced tendons was precipitated with ammonium sulfate and the dialyzed sample (see Materials and Methods) was placed on a 4-mL column containing concanavalin A–agarose. As indicated in Figure 2, most of the protein applied to the column passed directly through. A small amount of enzymic activity was eluted while washing the column but considerably more enzymic activity was recovered by elution of the column with 0.3 M α-methyl D-mannoside. The total recovery of activity from the column was 80%. Increasing the concentration of α-methyl D-mannoside to 0.5 M or eluting the column with a buffer containing both 0.3 M α-methyl D-mannoside and 30% ethylene glycol did not improve the yield. The small, second peak of enzymic activity shown in Figure 2 was not consistently observed.

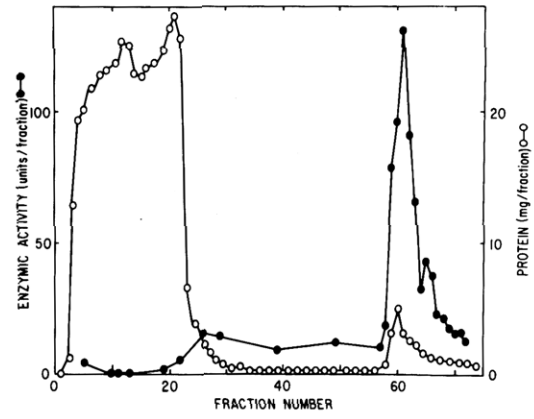

FIGURE 2: Chromatography of N-protease activity on a 4-mL concanavalin A–agarose column. An extract from chick embryo tendons was precipitated with ammonium sulfate and 40 mL of sample with a protein concentration of 15.8 mg/mL was passed through the column with a flow rate of about 4 mL/h. The column was washed first with the enzyme buffer then eluted with the same buffer containing 0.3 M α-methyl D-mannoside. Fractions of 2 mL were collected. Symbols: (●—●) enzymic activity; (○—○) protein concentration.

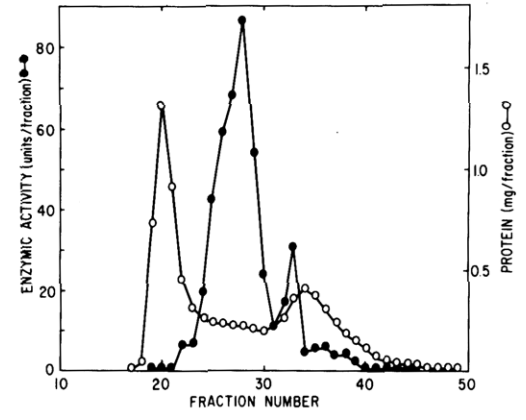

FIGURE 3: Gel filtration of N-protease activity on Ultrogel AcA 34 as described in text. Fractions of 2 mL were collected. Symbols: (●—●) enzymic activity; (○—○) protein concentration.

The enzyme recovered from the concanavalin A–agarose column was concentrated by precipitation with ammonium sulfate and then chromatographed by gel filtration. Most of the enzymic activity was recovered in the middle of the chromatograph and between the two major peaks of protein (Figure 3). As indicated, a small peak of enzymic activity eluted just after the major peak.

The purification procedures (Table II) provided a 300-fold purification of the enzymic activity. The recovery of activity was about 46%.

Characterization of Partially Purified N-Protease. Standardization of the gel filtration column with globular proteins indicated that enzyme in the major peak was about 260 000 daltons, and the enzyme in the minor peak was about 98 000 daltons.

The pH optimum of the partially purified enzymic activity was 7.4, 50% of the activity being observed at pH 6.6 and pH

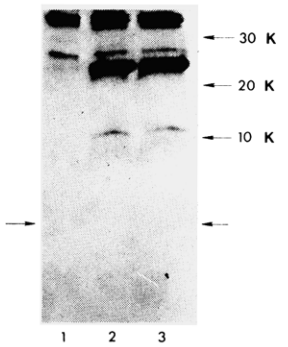

FIGURE 4: Polyacrylamide gel electrophoresis of reaction products on 20% gels in the presence of $NaDodSO_4$. About 4 μg of [^{14}C]proline-labeled type I procollagen incubated at 30 °C for 120 min without enzyme (slot 1). Same substrate incubated under same conditions with 0.3 unit of N-protease (slots 2 and 3). Gel was standardized with globular protein standards. All samples were reduced with 5% (v/v) β-mercaptoethanol.

FIGURE 5: Cleavage of type II procollagen by N-protease. Cleavage products on a 6% polyacrylamide gel in the presence of $NaDodSO_4$. About 35 000 cpm of [^{14}C]proline-labeled type II procollagen was incubated at 30 °C for 90 min without enzyme (slot 1), with 0.2 unit of N-protease (slot 2), and 0.4 unit of N-protease (slot 3).

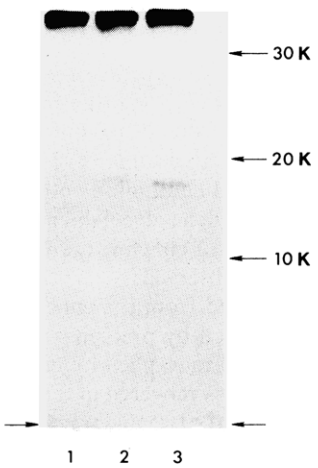

FIGURE 6: Cleavage of type II procollagen by N-protease. Cleavage products on a 20% polyacrylamide gel in the presence of $NaDodSO_4$. About 35 000 cpm of [^{14}C]proline-labeled type II procollagen was incubated at 30 °C for 120 min without enzyme (slot 1), with 0.2 unit of N-protease (slot 2), and 0.4 unit of N-protease (slot 3). Gel was standardized with globular proteins. All samples were reduced with 5% (v/v) β-mercaptoethanol.

8.8. No activity was obtained at pH 5.6. When the reaction was carried out at pHs below 5, there was a marked increase in the counts recovered in the ammonium sulfate supernate with the rapid assay. Examination of the reaction products on polyacrylamide gels suggested that the C-propeptide was removed but there was also degradation of the collagen region of the molecule (not shown). These observations were not pursued further.

Dialysis of the partially purified enzyme against 10 mM EDTA inhibited all enzymic activity. Activity was partially restored when the EDTA-treated enzyme was first dialyzed against 1 M KCl and 0.1 M Tris-HCl buffer, and then Ca^{2+}, Mg^{2+}, or Mn^{2+} was added to the reaction system. With either 0.5 or 5 mM Ca^{2+}, about 30% of the initial activity was restored. With the 5 mM Mg^{2+} or Mn^{2+}, 15–20% of the initial activity was restored. The enzyme remained inactive in the presence of 5 mM Fe^{2+}.

Characterization of Cleavage Products. As indicated in Figure 1, the large fragments obtained after cleaving procollagen with the N-protease comigrated with pCα1 and pCα2 chains of procollagen. The reaction products did not contain any measurable amounts of pNα1 or pNα2 chains, which have a greater mobility than the pCα chains (Olsen et al., 1976). Also, the reaction products did not contain any measurable amount of α1 or α2 chains.

The smaller fragments released during the enzymic reaction migrated at the dye front when the reaction products were examined on 6% polyacrylamide gels. When the same reaction products were examined on 20% polyacrylamide gels, two fragments were seen (Figure 4). The larger fragment had an apparent size of about 23 000 daltons compared with standards of globular proteins. The smaller fragment had an apparent size of about 11 000 daltons. As indicated in Figure 4, the amount of ^{14}C label in the larger peptide was greater than in the smaller peptide. Under the conditions employed here, there was no evidence of any other fragments either within the gels or at the dye front.

Cleavage of Type II Procollagen by the Partially Purified N-Protease. Type II procollagen was isolated from the medium of matrix-free cartilage cells using the procedure described previously (Dehm & Prockop, 1973) and the protein was then used as a substrate for the partially purified N-protease. As indicated in Figure 5, examination of the reaction products on 6% gels demonstrated that the proα1 chain of the type II procollagen was cleaved to a polypeptide of about the mobility expected for a pCα chain. The change in mobility was slightly less than the change seen with the cleavage of the proα1 type I chains to the pCα1 chains (compare Figure 5 with Figure 1). Examination of the reaction products on 20% acrylamide gels (Figure 6) demonstrated that the second fragment produced by the enzymic cleavage was about 18 000 daltons.

Effect of Substrate Conformation. As indicated above, the substrate employed for the N-protease reaction consisted of native type I or type II procollagen. To examine the effect of substrate conformation, type I and II procollagens were heat denatured at 55 °C for 10 min. The proteins were then immediately cooled on an ice bath and used for the enzymic reaction. As indicated in Figure 7, heat denaturation in itself gave rise to polypeptides smaller than proα1 and proα2 chains and some of these migrated with the pCα1 and pCα2 chains. It was apparent, however, that the heat-denatured type I procollagen was not cleaved by the enzyme as readily as the native protein (Figure 7). Similar results were obtained when native and heat-denatured type II procollagen were compared (not shown).

Discussion

The neutral N-protease examined here appears to be similar or identical to the enzymic activity previously obtained by Kohn et al. (1974) from calf tendon. Kohn et al. purified the

activity 2000-fold if calculated on the basis of the activity of whole tendon and about 30-fold if purification was calculated, as was done here, on the basis of the activity in the initial tissue extract. The N-protease from calf tendon cleaved the N-propeptides from both the pNα1 and pNα2 chain of pNcollagen en bloc in a manner similar to that observed here with the N-protease from chick embryos. The N-protease from calf tendon eluted in several peaks from a Sephadex G-200 column but most of the activity appeared in the void volume, suggesting that the enzyme was of relatively large size. Because intact procollagen was not available, the enzyme was not tested for C-protease activity.

With the rapid assay developed here, the N-protease from chick embryo tendons was purified about 300-fold and partially characterized. The major peak of the partially purified enzyme had an apparent molecular weight of 260 000 but, since a peak with an apparent molecular weight of 98 000 was also obtained, it is possible that most of the enzyme was isolated in an aggregated form. As suggested by previous observations (Bornstein et al., 1972; Layman & Ross, 1973; Kohn et al., 1974; Uitto, 1977), the N-protease required Ca^{2+} for activity but the requirement for Ca^{2+} was not highly specific in that it was partially replaced by Mn^{2+} or Mg^{2+}. Since the enzyme was bound to concanavalin A-agarose, it is presumably a glycoprotein.

The specificity with which the N-protease cleaved procollagen was not established definitively but the results suggested that proteolysis occurred at the same N-terminal site at which procollagen is cleaved in vivo. The larger fragments obtained were the same size as the pCα1 and pCα2 chains. One of the smaller fragments obtained was about the same size as the N-propeptide from the pNα1 chain of dermatosparactic calves (Kohn et al., 1974; D. Hörlein & P. Fietzek, in preparation) or sheep (Becker et al., 1976). The other small fragment obtained was of about the same size as the N-propeptide from the pNα2 chain of dermatosparactic calves (Kohn et al., 1974; H. Bentz, D. Hörlein, & P. Fietzek, in preparation) and sheep (Becker et al., 1976). The conclusion that the N-propeptide of the proα2 chain is smaller than the same propeptide from the proα1 chain was also supported by the observation that removal of this propeptide did not increase the mobility of the proα2 chain as much as removal of the N-propeptide from the proα1 chain. It should be noted, however, that Smith et al. (1977) obtained a collagenase-resistant fragment from the pNα2 chain of rat skin which was of about the same size as the comparable fragment from the proα1 chain. Their results suggest, therefore, that the N-propeptide from the proα2 chain of type I procollagen from rat is larger than the same N-propeptide from calves, sheep, or chick embryos.

The conclusion that the partially purified N-protease cleaved procollagen specifically was also supported by the observation that heat-denatured procollagen was cleaved much more slowly than native procollagen. Kohn et al. (1974) made a similar observation in that they found that the N-protease from calf tendon cleaved native pNcollagen more readily than isolated pNα1 and pNα2 chains. It appears therefore that correct conformation of the cleavage site is important for the proteolytic attack on the N-propeptides of procollagen. A similar situation appears to hold for some animal collagenases in that these enzymes hydrolyze triple-helical collagen more readily than α chains (Harris & Krane, 1972; McGroskery et al., 1973).

Of special interest was the observation that the partially purified N-protease cleaved type II procollagen. Although the N-protease was not purified to homogeneity, it is unlikely that the preparation contained a specific N-protease for type II procollagen since chick embryo tendons, from which the enzyme was extracted, do not synthesize type II collagen. It appears, therefore, that either the N-protease from chick embryo tendons has a broad specificity or the N-terminal cleavage site in type II procollagen is similar in structure to the N-terminal cleavage site in type I procollagen.

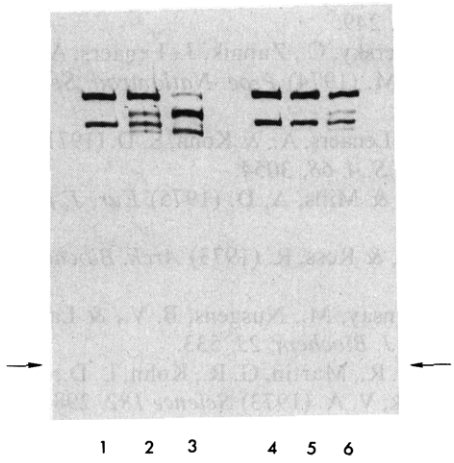

FIGURE 7: Effect of heat denaturation of substrate. Type I procollagen was heat denatured at 55 °C for 10 min and then used as a substrate for the enzymic reaction carried out at 30 °C for 90 min. The reaction products were reduced and examined on 6% polyacrylamide gel electrophoresis. About 2 μg of native [^{14}C]proline-labeled procollagen was incubated without enzyme (slot 1), with 0.1 unit (slot 2), and with 0.3 unit (slot 3) of N-protease. About 2 μg of heat-denatured procollagen incubated without enzyme (slot 4), with 0.1 unit (slot 5), and with 0.3 unit (slot 6) of N-protease.

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Photochemical Cross-Linking Studies on the Interaction of *Escherichia coli* RNA Polymerase with T7 DNA†

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ABSTRACT: We have identified the subunits of *Escherichia coli* RNA polymerase which are in close contact with the T7 phage DNA template using photochemical cross-linking. In nonspecific T7 DNA–enzyme complexes which occur in all regions of the DNA, subunits σ, β, and β' were cross-linked to the DNA. In contrast, in specific binary complexes which presumably occur at promoter sites, and in the initiation complex (holoenzyme + T7 DNA + initiator dinucleotides + three nucleoside triphosphates), only σ and β were cross-linked to DNA, while cross-linking of β' could not be demonstrated. These results (1) do not support the idea that α subunits are involved in the enzyme–template interaction, (2) raise the possibility that σ subunit participates directly in promoter recognition even though isolated σ does not bind to DNA, and (3) indicate different modes of interaction between RNA polymerase and DNA in nonspecific and specific complexes. These findings are relevant to the mechanism by which RNA polymerase carries out selective transcription.

The first step in gene transcription involves the interaction of RNA polymerase with the DNA template. This interaction leads to the formation of a binary complex which is able to bind substrates and initiate RNA synthesis. Two types of binary complexes can be formed: "specific" complexes at discrete sites on the template and "nonspecific" complexes which occur in all regions of the DNA, probably due to electrostatic interactions. In systems where RNA polymerases carry out selective transcription, specific binary complexes have been shown to form primarily at promoter regions of the DNA template, which contain the start signals for correct RNA synthesis (Chamberlin, 1976). The nonspecific binding of RNA polymerase to DNA is thought to play a role in facilitating promoter site selection, the step at which part of the cellular regulation of transcription takes place (von Hippel et al., 1974).

In order to understand the molecular mechanism of transcription, it is essential to know how RNA polymerase selects and recognizes promoter sites on the DNA template. A good system for studying the mechanism of promoter site selection is the transcription of bacteriophage T7 DNA. In vivo and in vitro transcription of T7 DNA by *E. coli* RNA polymerase starts near the 3′ end of the r strand (Summers & Siegel, 1969), where three major promoters have been mapped (Dunn & Studier, 1973; Minkley & Pribnow, 1973). Visualization by electron microscopy has located RNA polymerase on these promoters at positions which agree with those determined by transcription studies (Darlix & Dausee, 1975). Little is known, however, concerning the specific interactions that allow RNA polymerase to distinguish promoter sites from the numerous nonspecific binding sites along the DNA.

In this communication, we describe the spatial relationship of the different subunits of RNA polymerase relative to DNA in both specific and nonspecific enzyme–template complexes. Photochemical cross-linking was used to identify the subunits of the enzyme which are in close contact with the DNA template. This technique has recently been applied to investigate the interaction between *lac* repressor and *lac* operator DNA

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