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Isolation and Partial Characterization of the Carboxy-Terminal Propeptide of Type II Procollagen from Chick Embryos†

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ABSTRACT: The carboxy-terminal propeptide from type II procollagen was isolated from organ cultures of sternal cartilages from 17-day-old chick embryos. The procedure provided the first isolation of the carboxy-terminal propeptide in amounts adequate for chemical characterization. The propeptide was isolated as a disulfide-linked trimer with an apparent molecular weight of about 100 000. After reduction, monomers of about M_r 34 000 were obtained. Antibodies were prepared to the propeptide and used to establish its identity. The antibodies precipitated type II procollagen but did not precipitate type II procollagen from which the amino- and carboxy-terminal propeptides were removed with pepsin. No collagen-like domain was found in the propeptide, and the amino acid composition was similar to that of globular proteins. The circular dichroism spectrum of the propeptide suggested the presence of β-structure together with some random-coil structure. The data demonstrated that the type II carboxy-terminal propeptide is similar to the two different carboxy-terminal propeptides of type I procollagen in amino acid composition, molecular size, optical properties, and antigenicity. The homology among the type I and type II carboxy-terminal propeptides is consistent with the current hypothesis that they serve similar functions in vivo. The differences in structure may account for the selection of the appropriate proα chains to form the correct trimers in cells synthesizing several types of proα chains simultaneously.

The three major interstitial collagens, types I, II, and III, are first synthesized as procollagens which contain additional amino acid sequences at both the N- and C-terminal ends of the three proα chains of the molecules (Prockop et al., 1976; Fessler & Fessler, 1978; Bornstein & Traub, 1979; Bornstein & Sage, 1980; Davidson & Berg, 1981; Olsen, 1981a). Both the N- and C-propeptides[1] are cleaved in the conversion of the procollagens to their corresponding collagens. The N- and C-propeptides of several of these type-specific procollagens have been characterized. The primary structure was determined for the N-propeptide of the proα1(I) chain from calf (Hörlein et al., 1979), sheep (Rohde & Timpl, 1979), and chick (Pesciotta et al., 1980) and for the N-propeptide of the proα1(III) chain from calf (Timpl & Glanville, 1981; A. Brandt, D. Hörlein, P. Bruckner, R. Timpl, P. P. Fietzek, and R. W. Glanville, unpubished results). The N-propeptides of the proα2(I) chain from sheep (Becker et al., 1977), chick embryos (Tuderman et al., 1978), and rat (Smith et al., 1977)

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[1] Abbreviations: N-propeptide, amino-terminal propeptide of procollagen; C-propeptide, carboxy-terminal propeptide of procollagen; CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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were partially characterized. Also, the N-propeptide of the proα1(II) chain from chick embryos was recently isolated and partially characterized (Curran & Prockop, 1982). These four types of N-propeptide vary in structure, but all contain collagen-like domains.

The complete primary structures of the two C-propeptides of type I procollagen from chick embryos were determined by amino acid sequencing and by nucleotide sequenching (Showalter et al., 1980; Fuller & Boedtker, 1981; Dickson et al., 1981; Olsen, 1981a; Pesciotta et al., 1981). The C-propeptides from the proα1(I) and proα2(I) chains showed extensive homology but were not identical. Each contains about 246 amino acid residues and a single site at which carbohydrate is attached. The C-propeptides are linked by interchain disulfide bonds (Olsen et al., 1977).

To date, the C-propeptides from the other types of procollagens have not been characterized extensively. After bacterial collagenase digestion of type II procollagen (Merry et al., 1976; Fessler & Fessler, 1976; Olsen et al., 1976), a disulfide-linked trimer of about the same size as the trimer of the C-propeptide of type I procollagen was obtained. By gel electrophoresis, the monomers generated by reduction of the trimers of both these types of C-propeptides appeared to be identical. Similar observations were made with the disulfide-linked trimer obtained by collagenase digestion of type III procollagen from chick embryos (Fessler & Fessler, 1978).

We here report the first isolation of the C-propeptide of type II procollagen in amounts adequate for chemical characterization. To obtain the C-propeptide, we employed an organ culture system consisting of sternal cartilages from 17-day-old chick embryos, a system previously shown to synthesize type II procollagen in vitro (Dehm & Prockop, 1973; Uitto, 1977; Linsenmeyer et al., 1979) and a system we have recently employed as a source of type II N-propeptide (Curran & Prockop, 1982).

Experimental Procedures

Materials

Unless otherwise indicated, materials were obtained from sources previously specified.

Methods

Purification of the Type II C-Propeptide. Type II propeptides were isolated by a modification of the organ culture system which was developed by Olsen et al. (1977) and which was recently employed to prepare the type II N-propeptide (Curran & Prockop, 1982). Sternums were dissected from 40 dozen 17-day-old chick embryos and cleaned of adhering perichondrial tissue. The whole sterna were incubated in Dulbecco's modified Eagle's medium supplemented with 40 μg/mL sodium ascorbate, 60 μg/mL β-aminopropionitrile, and 1–2 μCi/mL ^{14}C amino acid mixture. The incubations were carried out for 24 h in a 10% CO_2/90% air atmosphere at 37 °C with shaking. The medium was supplemented with streptomycin/penicillin to final concentrations of 100 units/mL and 100 μg/mL, respectively.

At the end of the incubation period, the medium was separated from the sternums by centrifugation and cooled to 4 °C. Proteinase inhibitors were added to give final concentrations of 24 mM EDTA, 10 mM *N*-ethylmaleimide, 1 mM *p*-aminobenzamidine, and 1 mM phenylmethanesulfonyl fluoride (Hoffmann et al., 1976).

The first step in the purification consisted of chromatography on a column of DEAE-cellulose under the same conditions used to purify the type II N-propeptides (Curran & Prockop, 1982). The medium was dialyzed against a 0.02 M Tris-HCl buffer, pH 8.6 at 23 °C, containing deionized 2 M urea. The dialyzed medium was applied to a 2.5 × 20 cm DEAE-cellulose column and eluted with an 800-mL linear gradient of 0–0.35 M NaCl (Curran & Prockop, 1982). Flow rates were 120–140 mL/h. Five-milliliter fractions were collected and assayed by liquid scintillation counting.

The first peak eluted in the NaCl gradient from the DEAE-cellulose column contained the type II C-propeptide. This material was further purified either by chromatography on CM-cellulose or on a column of concanavalin A linked to agarose.

For chromatography on CM-cellulose, the fractions in the first peak from the DEAE column were pooled, desalted on a 4 × 50 cm Bio-Gel P-2 (200–400 mesh) column equilibrated in 0.2 M NH_4HCO_3, and lyophilized. The samples were dissolved in a 0.1 M sodium acetate buffer, pH 3.6 at room temperature, containing 8.0 M deionized urea. The dialyzed samples were then applied to a 1.5 × 1.5 cm column of CM-cellulose. The column was eluted at room temperature with a 400-mL gradient of 0–0.2 M NaCl. The flow rate was 80 mL/h, and 5.0-mL fractions were collected. Fractions were assayed for radioactivity, and the fractions containing the C-propeptide were dialyzed against 0.2 M ammonium bicarbonate, lyophilized, and stored frozen.

To purify the C-propeptide on the column of concanavalin A linked to agarose, the first peak eluted from the DEAE column was desalted on the Bio-Gel P-2 column, lyophilized, and dialyzed against 0.1 M Tris-HCl buffer, pH 7.8 at room temperature, containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. The sample in a volume of about 10 mL was then applied to a 0.5 × 3.5 cm column of concanavalin A–agarose (Con A–Sepharose; Pharmacia) by recycling of the sample through the column for 15 h at a flow rate of about 10 mL/h. The column was washed with about 10 column volumes of the sample buffer and eluted with a linear gradient of 0–0.5 M methyl α-mannoside in 0.1 M Tris-HCl buffer, pH 7.8, containing 5 mM EDTA. The total volume of the gradient buffer was 300 mL, and fractions of 1 mL were collected at a flow rate of 10 mL/h. Fractions were assayed for radioactivity. The peak eluted from the column was dialyzed against 0.2 M ammonium bicarbonate, lyophilized, and stored frozen.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed by using standard procedures. Routinely, slabs of 2-mm thickness were used with polyacrylamide concentrations of 6% or 15%. For fluorography, gels were impregnated with 2,5-diphenyloxazole, dried, and exposed to RP Royal X-OMAT X-ray film (Kodak) at −70 °C (Bonner & Laskey, 1974; Laskey & Mills, 1975). For protein staining, gels were immersed in a solution of 20% trichloroacetic acid and 0.025% Coomassie Brilliant Blue R for 1 h at room temperature. Gels were destained in 7.5% acetic acid and 15% methanol.

Collagenase Digestion. Lyophilized propeptide samples were dissolved in and dialyzed against 0.4 M NaCl in 0.1 M Tris-HCl buffer, pH 7.4 at 4 °C, to which *N*-ethylmaleimide and $CaCl_2$ were added to final concentrations of 2.5 and 5.0 mM, respectively. The concentration of propeptides was usually 50–100 μg/mL. One hundred units per milliliter bacterial collagenase (form III; Advance Biofactures Corp.) was added, and the sample was incubated for 6 h at 37 °C. At the end of the incubation, 3 volumes of polyacrylamide gel sample buffer containing 5 mM EDTA was added to the sample which was then boiled and applied to a 15% polyacrylamide gel.

Amino Acid Analysis. Type II C-propeptide was prepared for amino acid analysis by dialysis against 0.1 N acetic acid,

lyophilization, and hydrolysis under N_2 in 6 N HCl for 16 h at 116 °C. The amino acid analyzer was a Beckman Model 121B. The propeptide residues were prepared as described previously (Curran & Prockop, 1982) for determination of methionine as methionyl sulfoxide and sulfone and for determination of cysteine as cysteic acid.

CD Studies. The CD spectrum of the C-propeptide was measured in a Varian Model 61 spectropolarimeter and in water-jacketed cells. The temperature was controlled by a water bath with an automatic programmer (Neslab Instrument, Inc., Model TP-2), and the temperature was recorded by a thermistor inserted into the cell. Cells with 2-mm path lengths were used, and the propeptide concentration was 20–30 μg/mL. To ensure that the propeptide did not precipitate during the measurements, 10-μL aliquots from the top of the cuvette were assayed for ^{14}C before and after each measurement. There was no apparent loss of labeled propeptide during the CD measurements.

Pepsin and CNBr Digestion. For pepsin digestion, [^{14}C]-procollagen samples containing about 200 μg of [^{14}C]procollagen were dialyzed against 0.1 N acetic acid overnight at 4 °C. The pepsin solution was prepared by dissolving 1 mg of lyophilized pepsin (Boehringer-Mannheim) in 1 mL of acetic acid. This pepsin solution was added to 10 mL of procollagen, and the sample was incubated for 2 h at 18 °C. The reaction was stopped by the dropwise addition of 0.5 N NaOH and titration to pH 7.4. The sample was dialyzed overnight at 4 °C against a 0.1 M Tris-HCl buffer, pH 7.4 at 4 °C, containing 0.4 M NaCl. Aliquots of the procollagen digest were boiled in a final concentration of 4% $NaDodSO_4$ for 3 min, dialyzed into slab sample buffer, and applied to 6% polyacrylamide slab gels for visualization.

CNBr digestion was performed with a modification of the method of Epstein (1974). The lyophilized C-propeptide was dissolved in 70% formic acid and flushed with nitrogen for 15 min. A 1000-fold molar excess of recrystallized CNBr was added to the samples which were flushed with nitrogen for another 15 min. The incubation was then continued at 37 °C for 4 h.

Immunological Technique. Antibodies to the type II C-propeptide were prepared by intradermal injections of the purified peptide into white New Zealand male rabbits. The propeptide was dissolved in phosphate-buffered saline, pH 7.4, and emulsified in Freund's complete adjuvant. Booster injections in incomplete adjuvant were given at 14–17-day intervals.

Binding radioimmune assays were used to titer the immune sera developed in the rabbits. The immune complexes were precipitated with either protein A–Sepharose 4B (Pharmacia) or sheep anti-rabbit IgG (Nist et al., 1975; Olsen et al., 1977). The sheep anti-rabbit IgG was titrated to achieve optimal precipitation of the immune complexes. Immune sera were incubated with their respective antigens for 1 h at room temperature. The protein A–Sepharose or the second antibody was added, and the incubation was continued at 4 °C for 24 h. The immune complexes were washed, extracted into 0.2 N HCl, and counted with Aquasol (New England Nuclear) as scintillation cocktail. Alternatively, the complexes were boiled in 200 μL of a buffer containing 4% $NaDodSO_4$, 0.125 M Tris-HCl (pH 6.8), 10% glycerol, 0.025% bromophenol blue, and 10 μL of 2-mercaptoethanol. The boiled samples were dialyzed against this buffer and applied to a 15% polyacrylamide slab gel for visualization.

Results

Isolation of C-Propeptide. Sternal cartilages were incubated in vitro under the same conditions used previously to obtain type II N-propeptide (Curran & Prockop, 1982). As reported previously [see Figure 1 in Curran & Prockop (1982)], the protein subsequently identified as the type II C-propeptide was recovered in the first peak when incubation medium from sternal cartilages was applied to a DEAE-cellulose column and the column was eluted with a linear salt gradient. The first peak from the DEAE column was pooled, desalted, and then further purified by one of two alternative procedures.

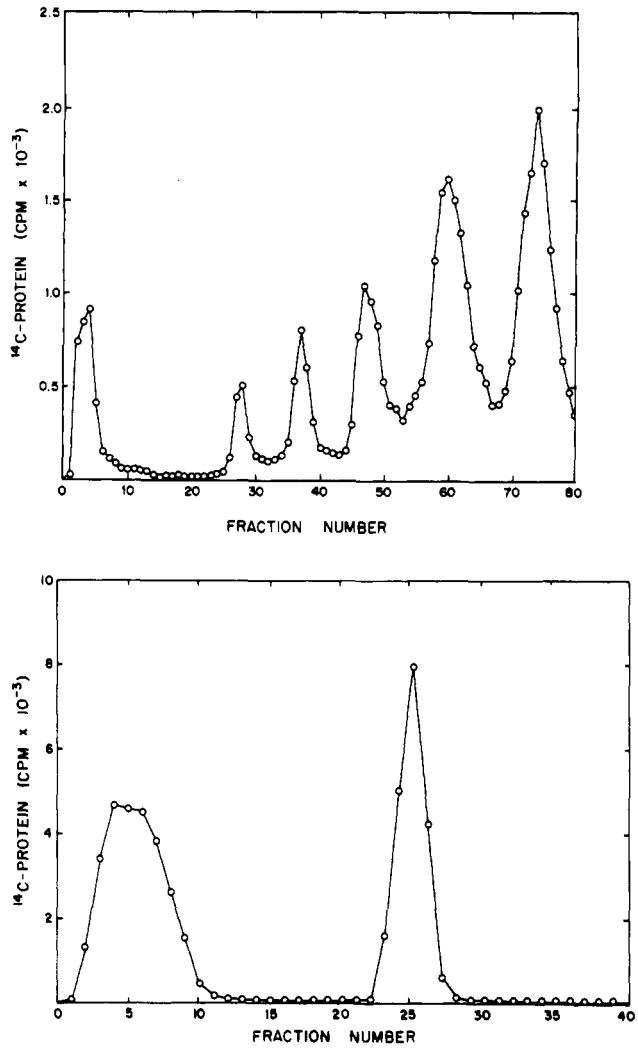

FIGURE 1: (Top panel) CM-cellulose chromatography of peak 1 material from the DEAE-cellulose column containing type II C-propeptide. The column conditions are described in the text; the gradient begins at fraction 10. The last peak eluted by the gradient is the C-propeptide. (Bottom panel) Chromatography on concanavalin A linked to agarose of peak 1 material. Conditions are described in the text. The gradient was begun at fraction 15. The C-propeptide is eluted in the gradient.

In the first procedure, the material was further purified by chromatography on a column of CM-cellulose in buffer containing 8 M urea. Five peaks of radioactivity were eluted from the column (upper panel, Figure 1). Fractions 36–40 contained N-propeptide, while fractions 71–79 contained the C-propeptide. Gel electrophoresis in $NaDodSO_4$ showed that the N-propeptide (lane 2, upper panel in Figure 2) and the C-propeptide (lane 4, upper panel in Figure 2) were radiochemically homogeneous. When the C-propeptide was examined without reduction, it migrated as a protein with the apparent molecular weight of about 100 000 (not shown). After reduction, it migrated as a single band with the apparent molecular weight of about 34 000 (lane 4, upper panel in Figure 2). The migration of the unreduced propeptide was

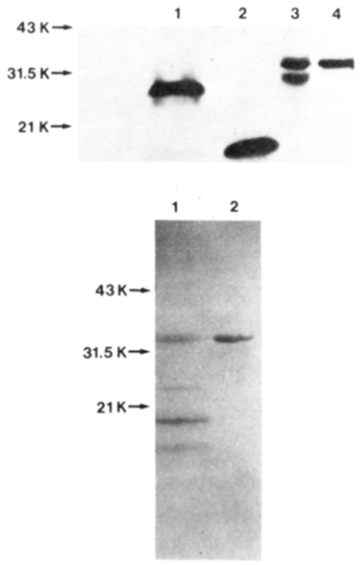

FIGURE 2: (Top panel) Fluorogram of fractions eluted with gradient from the CM-cellulose column (top panel, Figure 1). (Lane 1) Unknown peptide which is the first peak eluted in the gradient. (Lane 2) Type II N-propeptide eluted as the second peak in the gradient. (Lane 3) Unknown peptides eluted in the fourth peak. (Lane 4) Type II C-propeptide eluted as the fifth peak. (Bottom panel) Protein staining of polyacrylamide gel of type II C-propeptide eluted from the Con A–Sepharose column (bottom panel, Figure 1). (Lane 1) Breakthrough fractions of the Con A–Sepharose column. (Lane 2) Type II C-propeptide eluted by gradient from Con A–Sepharose.

the same as that of the trimer of the C-propeptide of type I procollagen from chick embryos. The reduced C-propeptide migrated with the same mobility as the C-propeptide from the proα1(I) chain of type I procollagen from chick embryos.

On the basis of assays of ^{14}C-labeled material, the recovery from the DEAE column was 80%, the recovery from the P-2 desalting column was 85%, and the recovery from the CM-cellulose column (Figure 1) was 70%. The C-propeptide accounted for 40% of the ^{14}C in the CM-cellulose chromatogram. The overall recovery therefore was about 48%, and the procedures yielded about 4 μg of C-propeptide per chick embryo sternum.

In the alternative procedure, the peak eluted from the DEAE-cellulose column was desalted and chromatographed on a column of concanavalin A–Sepharose (lower panel, Figure 1). The recoveries were about 50%, or the same as with the first procedure. The C-propeptide obtained with the second procedure was also homogeneous (lane 2, lower panel in Figure 2).

Characterization of the Type II C-Propeptide with Antibodies. Antibodies were raised in rabbits by repeated intradermal injections of the purified type II C-propeptide. The antisera against the C-propeptide reacted with about 90% of ^{14}C-labeled C-propeptide (Figure 3). The antibodies were prepared with type II C-propeptide isolated with the first procedure described above, but identical results were obtained when the antibodies were reacted with ^{14}C-labeled C-propeptide isolated with either of the two procedures.

The same antisera reacted with about 80% of ^{14}C-labeled type II procollagen (Figure 3). Failure to achieve complete reaction with the corresponding ^{14}C-labeled procollagen was previously observed with antibodies against the type I C-propeptide (Olsen et al., 1977) and antibodies against the type II N-propeptide (Curran & Prockop, 1982). Similarly, a previous report indicated that monoclonal antibodies to type I collagen did not fully react with type I collagen (Linsenmayer et al., 1979). The antiserum reacted with about 15% of type

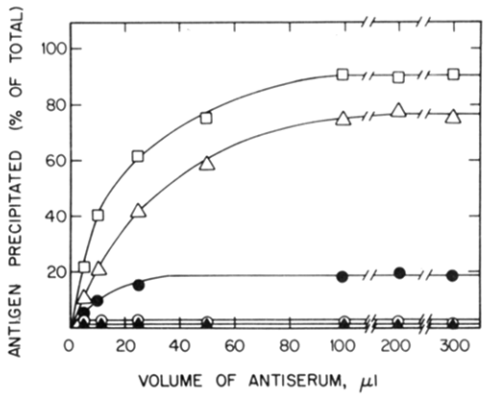

FIGURE 3: Binding radioimmune assay using antiserum against type II C-propeptide. About 6000 cpm of type II C-propeptide (□), 5000 cpm of type II procollagen (△), 9000 cpm of type I procollagen (●), 4000 cpm of type II N-propeptide (○), and 8000 cpm of pepsin-treated type II procollagen were used (▲). Values are means from five or six experiments. The second antibody was 500 μL of sheep anti-rabbit IgG for most of the experiments, but 100 μL of protein A–Sepharose was used for the sample of type II N- and C-propeptide in some experiments. Values on abscissa indicate volume of antisera used for reaction with procollagens after dilution of antisera 1:10 with buffer. Undiluted antisera were used for reaction with propeptides.

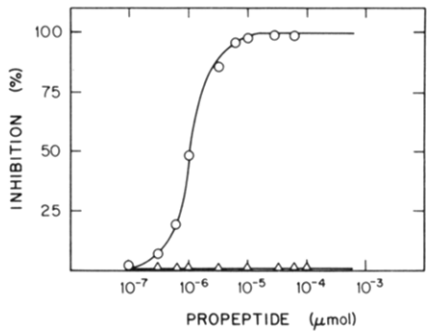

FIGURE 4: Inhibition radioimmune assay of type II procollagen. Fifty microliters of ^{14}C-labeled type II procollagen (5000 cpm) and 50 μL of antiserum to type II C-propeptide were used. Antisera were diluted 1:10 with buffer. The C-propeptide was labeled at low specific activity for purification but was sufficiently diluted in the assay so as not to affect the observed values for precipitation of the ^{14}C-labeled procollagen. The second antibody was 500 μL of sheep anti-rabbit IgG. Other conditions as described in the text. Type II C-propeptide (○); type II N-propeptide (△).

I procollagen under the same conditions (Figure 3). It did not react with the N-propeptide from type II procollagen; also, it did not react with type II procollagen that had been treated with pepsin to remove both N- and C-propeptides.

The antisera were also used for an inhibition radioimmune assay. As indicated in Figure 4, the isolated C-propeptide, at a concentration of 2×10^{-8} M, completely inhibited reaction with type II procollagen. As expected, the N-propeptide of type II procollagen had no effect.

Amino Acid Composition. Amino acid analysis of the type II C-propeptide demonstrated that the peptide contained no hydroxyproline or hydroxylysine (Table I). Therefore, there was no evidence of a collagen-like domain such as the domains found in the N-propeptides of type I, II, and III procollagens. Three residues of methionine per mol were present. In general, the composition was similar to that of the C-propeptide of the proα1 chain of type I procollagen. The type II C-propeptide, however, contained less aspartate, threonine, and arginine, and it contained more glutamate. Also, the type II C-propeptide contained no tyrosine.

Digestion with Bacterial Collagenase and CNBr. The absence of a collagen-like domain was confirmed by experiments

Table I: Amino Acid Composition of Type II C-Propeptide

amino acid	Proα1(II) C-propeptide[a]	Proα1(I) C-propeptide[b]	Proα2(I) C-propeptide[b]
4-Hyp	0	0	0
Asp	20	40	41
Thr	13	28	29
Ser	19	20	22
Glu	48	35	30
Pro	11	13	11
Gly	32	27	25
Ala	22	19	21
$^1/_2$-Cys	9	11	11
Val	21	16	12
Met	3	2.5	0.7
Ile	17	20	18
Leu	20	20	24
Tyr	0	11	8.5
Phe	8	9.5	11
Hyl	0	0	0
Lys	19	19	20
His	6	5.8	7.6
Arg	7	13	13

[a] Values are expressed as residues per peptide by assuming a molecular weight of 34 000. They are the means of seven analyses on five separate preparations. [b] Values for type I C-propeptides are from Olsen et al. (1977).

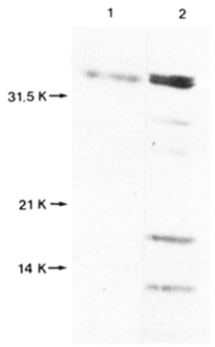

FIGURE 5: Fluorogram of 15% polyacrylamide gel in $NaDodSO_4$. Protein staining of the gel yielded identical results. (Lane 1) Collagenase digestion of type II C-propeptide. Conditions as described in the text. (Lane 2) CNBr digestion of the type II C-propeptide. Conditions as described in the text.

with bacterial collagenase. As indicated in lane 1, Figure 5, digestion of the C-propeptide with bacterial collagenase did not change the size of the peptide as judged by its electrophoretic mobility in $NaDodSO_4$.

After digestion with CNBr, two major fragments were obtained (lane 2, Figure 5). Since the C-propeptide contained three methionine residues, four CNBr peptides were expected. The two major fragments shown in Figure 5 had molecular weights of 16 000 and 12 000 and accounted for about 80% of the mass of the monomer. It is likely therefore that the two faint bands of about M_r 29 000 and 26 000 molecular weight shown in lane 2 of Figure 5 are partial cleavage products and that the other two expected fragments were small and lost in the course of processing the electrophoretic gels. The pattern of CNBr peptides was different from the pattern of either of two type I C-propeptides from chick embryos (Showalter et al., 1980; Pesciotta et al., 1980; Dickson et al., 1981).

CD Spectrum of the Type II C-Propeptide. The structure of the type II C-propeptide was also examined by CD (Figure 6). The spectrum had a negative peak at 215 nm with a specific residue ellipticity of about −5000 deg cm^2 dmol^{-1}. The negative peak at 215 nm suggested the presence of β-structure, but the specific residue ellipticity was considerably less than

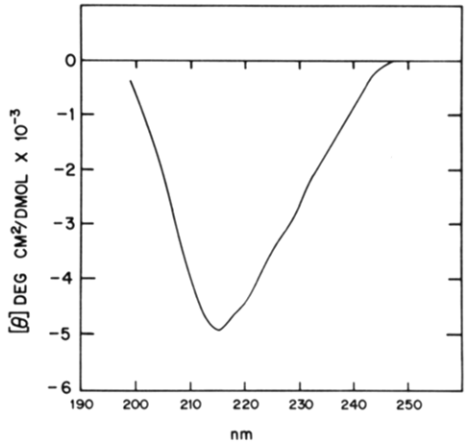

FIGURE 6: CD spectrum of type II C-propeptide. Twenty micrograms of C-propeptide was dissolved in 50 mM sodium phosphate buffer, pH 7.5. Spectrum taken at 20 °C in a 2-mm path-length cell.

that observed with pure β-structures, about −20 000 deg cm^2 dmol^{-1} (Cantor & Schimmel, 1980). The relatively small mean residue ellipticity of the 215-nm negative peak may be explained by contributions from random-coil structures which show a broad positive peak of about 15 000 deg cm^2 dmol^{-1} at about 220 nm (Cantor & Schimmel, 1980).

The CD spectrum of the type II C-propeptide is similar to the spectrum of the type I C-propeptide (T. Doering, P. P. Fietzek, E. Eikenberry, B. R. Olsen, D. M. Pesciotta, and B. Brodsky, unpublished results) both in shape and in mean residue ellipticity at about 215 nm.

Discussion

The type II C-propeptide isolated here was homogeneous as judged by gel electrophoresis. In initial experiments, the yields were low apparently because the propeptide was insoluble in most aqueous buffers. The procedures developed here, however, provided the first isolation of the propeptide in reasonable yields.

The data demonstrated that the type II C-propeptide is a globular structure. No collagen-like domain was detected, and the amino acid composition was similar to that of globular proteins. The CD spectrum suggested the presence of β-structure together with some random-coil structure.

On the basis of the data presented here, the type II C-propeptide is homologous with the two different C-propeptides of type I procollagen. The similarities include the amino acid compositions (Olsen et al., 1977), molecular size, CD spectra (T. Doering, P. P. Fietzek, E. Eikenberry, B. R. Olsen, D. M. Pesciotta, and B. Brodsky, unpublished results), and partial cross-reactivity with the antibodies prepared here. The type II C-propeptide, however, is not identical with either of the type I C-propeptides. The cross-reactivity with the antibodies was not complete, the pattern of CNBr peptides was different and, particularly noteworthy, the type II C-propeptide lacked tyrosine.

The homology among the type I and type II C-propeptides is consistent with the hypothesis that these propeptides serve similar functions in vivo. The nucleus for the folding of proα chains into a triple-helical conformation probably first forms near the C-terminal end of the α-chain domains after the C-propeptides have directed chain association and interchain disulfide bonds have been synthesized (Bächinger et al., 1980; Bruckner et al., 1981). The differences in the structure of the C-propeptides may account for selection of the appropriate proα chains to form the correct trimers in cells synthesizing type I procollagen or in cells synthesizing several types of

procollagen simultaneously (Rosenbloom et al., 1976; Prockop et a., 1976, 1979; Fessler & Fessler, 1978; Bornstein & Traub, 1979; Bornstein & Sage, 1980; Davidson & Berg, 1981; Olsen, 1981b; Timpl & Glanville, 1981).

Acknowledgments

We are grateful to Dr. Bjørn R. Olsen for many helpful discussions and suggestions during the course of this work. Dr. Robert Alper, Connective Tissue Institute, University of Pennsylvania, kindly performed all amino acid analyses. We thank Nancy Kedersha for invaluable assistance and suggestions.

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