Further Characterization of Procollagen. Purification and Analysis of the Proα1 Chain of Chick Bone Procollagen†

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ABSTRACT: Methods were developed for the preparative separation of the proα1 and proα2 chains of procollagen. Preparative acrylamide gel electrophoresis was used to obtain substantial purification of proα1. The amino acid composition of proα1, calculated for a chain of 1540 residues, agrees closely with the sum of previously determined compositions of the α1 chain, an NH_2-terminal extension of 200 residues, and a COOH-terminal extension of 340 residues. This result supports the conclusion that the proα1 chain contains precursor-specific regions at both the NH_2- and COOH-terminals and suggests a molecular weight of approximately 150,000 for the entire chain.

The biosynthetic precursor of type I collagen, procollagen, consists of two identical proα1 chains and a homologous proα2 chain. Procollagen differs from collagen in molecular weight, in the presence of interchain disulfide bonds and in other properties (for reviews see Schofield and Prockop, 1973; Bornstein, 1974; Martin et al., 1975). Early studies of procollagen, extracted from chick cranial bone with acetic acid, indicated a molecular weight of approximately 115,000 for the proα1 chain (Bornstein et al., 1972), in good agreement with a molecular weight of 20,000 determined for the non-triple-helical NH_2-terminal extension present in this chain (von der Mark and Bornstein, 1973). Similar molecular weights were estimated for the constituent chains of dermatosparactic procollagen, an intermediate in the conversion of procollagen to collagen which accumulates in cattle defective in the enzyme procollagen peptidase (Lenaers et al., 1971; Kohn et al., 1974).

It now appears that acid-extracted procollagen represents a truncated form of the precursor (Fessler et al., 1973; Monson and Bornstein, 1973) in which the NH_2-terminus may be intact but a disulfide-bonded COOH-terminal domain is missing (Tanzer et al., 1974; Murphy et al., 1975; Byers et al., 1975). In order to characterize the intact procollagen molecule, we have improved the procedure for the extraction of procollagen to minimize proteolysis. We report here methods for the separation of the constituent proα chains of procollagen and the amino acid composition of the proα1 chain.

Experimental Section

Materials. L-[2,3-^{3}H]Proline (43.1 Ci/mmol) was purchased from New England Nuclear. Pepstatin was obtained from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan). Sodium dodecyl sulfate was purchased from BDH Chemicals, Ltd. (Poole, England) and was further purified for use in preparative gel electrophoresis by passage through a cation exchange resin (AG 50W-X8, Bio-Rad Laboratories). *N,N'*-Diallyltartardiamide and acrylamide (electrophoresis grade) were purchased from Eastman. All other chemicals were reagent grade or the purest commercially available. An 8 *M* urea solution was deionized immediately before use on a mixed bed ion exchange resin (Bio-Rex RG 501-X8, 20–50 mesh, Bio-Rad Laboratories). The scintillant employed was 10% BioSolv 3 (BBS-3, Beckman) in toluene containing 4 g/l of Omnifluor. All glassware used in handling reduced and alkylated proα chains was siliconized with Siliclad (Clay-Adams).

Preparation of Procollagen. Cranial bones were surgically removed from 5 dozen 17-day-old chick embryos and placed directly in 62 ml of Dulbecco's modified Eagle's medium (GIBCO) containing 0.025 *M* Hepes buffer (pH 7.4) in place of bicarbonate. The medium was further modified by the addition of β-aminopropionitrile, sodium ascorbate, glutamine, penicillin, and streptomycin as described (Bornstein et al., 1972). After equilibration of the medium with O_2, the bones were incubated at 37° in sealed bottles for 1 hr in a shaking water bath. Thereafter, the bones were rinsed twice with 30 ml of medium to remove tissue debris and finally labeled for 18 min in 62 ml of fresh medium containing 16 μCi/ml of L-[2,3-^{3}H]proline. The incubation was terminated by washing the bones with distilled water at 0°. All subsequent procedures were performed at 0–4°.

Procollagen was extracted by immediate homogenization in 25 ml of 1 *M* NaCl, 0.05 *M* Tris-HCl (pH 7.5) containing 0.025 *M* EDTA, 0.01 *M* MalNEt, 1 m*M* Dip-F, and 1 m*M* benzamidine hydrochloride. An equal volume of extraction buffer was added and the homogenate was centrifuged immediately for 10 min at 39,000*g*. Procollagen and collagen were precipitated from the supernatant by increasing the NaCl concentration to 20% and stirring for 1 hr. The precipitate was collected by centrifugation for 20 min at 39,000*g*.

The pellet was dissolved by stirring for 1 hr in 20 ml of extraction buffer plus 0.001% pepstatin and the sample was clarified by centrifugation. The supernatant was precipitated by bringing the solution to 5% with Cl_3CCOOH. Precipitated samples were allowed to stand for 10 min before the

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[Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MalNEt, *N*-ethylmaleimide; Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethyanesulfonyl fluoride.

pellet was harvested by centrifugation at 10,000*g* for 10 min.

Reduction and Alkylation of Procollagen. Cl_3CCOOH-precipitated samples were dissolved by stirring for 30 min at 23° in 20 ml of 8 *M* urea, 0.5 *M* Tris-HCl (pH 8.5) containing 10 m*M* EDTA, 1 m*M* Dip-F, 1 m*M* benzamidine, and 0.001% pepstatin. After clarification by centrifugation, the sample was reduced under a N_2 atmosphere with 0.05 *M* dithiothreitol at 23° for 4 hr. The pH of the solution was reduced to 8.0 with NaOH and iodoacetic acid was added to a final concentration of 0.11 *M*. Alkylation was performed in the dark for 40 min at 23° and terminated by the addition of excess 2-mercaptoethanol.

Diethylaminoethylcellulose Chromatography. Reduced and carboxymethylated samples were dialyzed in the dark overnight against three changes of starting buffer, 0.025 *M* Tris-HCl (pH 8.5) containing 2 *M* urea, 0.1% 2-mercaptoethanol, 1 m*M* benzamidine, 1 m*M* $PhCH_2SO_2F$, and 2 m*M* EDTA. The sample was denatured at 40° for 20 min and centrifuged if necessary at 39,000*g* for 20 min at 40° to remove insoluble material. The sample was immediately applied to a freshly packed and equilibrated DEAE-cellulose column (2.5 × 15 cm) at 40°. The column was washed with 20 ml of starting buffer and then eluted with a linear gradient from 0 to 0.25 *M* NaCl over a total volume of 1200 ml at a flow rate of 220 ml/hr. Aliquots of 10-ml fractions were acidified with 0.01 ml of concentrated HCl and counted immediately in 10 ml of BBS-3 scintillant. Additional inhibitors were added to the tubes containing the radioactive peaks to double the concentration present in the elution buffer. Each peak was pooled, concentrated by vacuum dialysis overnight, rapidly dialyzed against 1 m*M* NH_4HCO_3, and lyophilized in freshly siliconized tubes for subsequent analysis.

Analytical Dodecyl Sulfate–Urea Gel Electrophoresis. Electrophoresis was performed according to the method of Goldberg et al. (1972). Gels were 5% in acrylamide and 0.07% in methylenebisacrylamide and were polymerized in electrophoresis buffer (0.1 *M* phosphate (pH 7.0) containing 0.1% dodecyl sulfate and 0.5 *M* urea). Lyophilized samples were dissolved in sample buffer: 1% dodecyl sulfate, 0.01 *M* phosphate (pH 7.0), 0.05 *M* urea containing 10% glycerol, 0.05 *M* dithiothreitol, and 0.002% Bromophenol Blue. The samples were heat-denatured for 20–60 min at 60°. Samples of 0.05–0.1 ml were applied to the gels and electrophoresis was performed at 15 mA/13-cm gel for 4 hr. The use of dansylated collagen standards, slicing, and digestion of gels have been described (Monson and Bornstein, 1973). Digested samples were counted in 12 ml of BBS-3 scintillant.

In some instances, gels were stained with Coomassie Blue (0.25% Coomassie Blue, 20% Cl_3CCOOH, 45% methanol, and 9% acetic acid) for 2 hr and destained in 5% methanol and 7.5% acetic acid.

Preparative Dodecyl Sulfate–Urea Gel Electrophoresis. The electrophoresis apparatus described by Hagen and Young (1974) was modified by the addition of a circulating water cooling jacket around the central gel tube (Savant Instruments, New York). Preparative gel electrophoresis was performed using the same buffer system described above for analytical gels except that *N,N'*-diallyltartardiamide was substituted for methylenebisacrylamide as a cross-linking reagent. Samples of proα1 were dissolved in sample buffer and incubated at 70° for 30–60 min. The sample was applied to a 3.3% acrylamide and 0.36% *N,N'*-diallytartardiamide gel (6.5 × 1.5 cm) which had been preelectrophoresed for 1.5 hr. No more than 6–8 × 10^6 cpm (0.5–1 mg total protein) could be successfully placed on the gel. Electrophoresis was performed at 20° with an initial current of 40 mA and a constant voltage of 35 V maintained throughout the run. The flow cell was eluted with electrophoresis buffer containing 1 m*M* $PhCH_2SO_2F$ at a rate of 5–6 ml/hr and 0.5-ml fractions were collected. Aliquots were acidified with 0.2 ml of 0.01 *N* HCl and then counted in 10 ml of BBS-3. Under these conditions, the yield of radioactivity from the gel was 50–60%. Pooled fractions were dialyzed against 1 m*M* NH_4HCO_3 prior to lyophilization; 90% of the eluted protein was recovered by this method.

The success of this method was dependent on meticulous attention to details. The apparatus, buffers, and all glassware were required to be scrupulously clean in order to avoid contamination of the eluted protein. Also, the dialysis casing used with eluted samples was preextracted with dodecyl sulfate containing buffer to remove traces of contaminating plant protein.

Amino Acid Analysis. Analyses were performed on chains isolated by preparative dodecyl sulfate acrylamide gel electrophoresis. Lyophilized samples were hydrolyzed for 24 hr in triply distilled, constant boiling hydrochloric acid. Hydrolysates were analyzed in duplicate with a Durrum analyzer, Model D-500, using both a regular program for protein hydrolysates and a special elution program which, in addition, separated 4-hydroxyproline and hydroxylysine from other amino acids. Provided that rigorous precautions were taken, reliable analyses could be made of samples with as little as 4 μg of protein.

Results

Efforts to separate the proα1 and proα2 chains of procollagen were plagued by two major problems. First, complete reduction and alkylation of the disulfide bonds in procollagen resulted in proα chains which bound irreversibly to glass surfaces and column supports such as agarose and carboxymethylcellulose even in the presence of 6–8 *M* urea or 5 *M* guanidinium hydrochloride. Attempts to apply established procedures for the separation of the α1 and α2 chains of collagen to the fractionation of procollagen chains were also unsuccessful. Second, the proα chains were extremely susceptible to limited proteolysis resulting in truncated forms of these chains. In most instances, when reasonable yields were obtained after column chromatography, analysis of the eluted chains by dodecyl sulfate gel electrophoresis revealed shortened proα chains equal to or somewhat larger than proα chains derived from acid-extracted procollagen. These problems were largely overcome when the cysteine residues in the proα chains were carboxymethylated with iodoacetic acid, all steps were carried out in neutral or basic buffers, protease inhibitors were included during each step of the purification, and the purification was carried out as rapidly as possible.

When reduced and carboxymethylated procollagen was chromatographed on DEAE-cellulose at 40°, pH 8.5, in the presence of protease inhibitors two major peaks of radioactivity were obtained (Figure 1). The counts in the second peak were twice that in the first, suggesting that the second peak contained proα1 and the first proα2. This interpretation was verified by a comparison of dodecyl sulfate gels of an aliquot of the sample applied to the column with gels of the material eluting in each of the two peaks (Figure 2). As expected, the sample applied contained proα1 and proα2 in

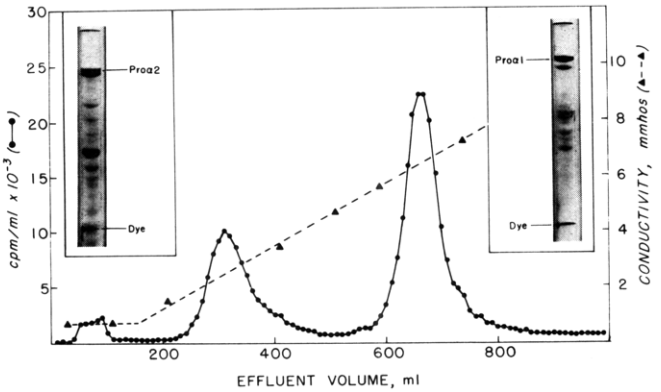

FIGURE 1: DEAE-cellulose chromatography at 40° of a 1 *M* NaCl extract of [^{3}H]proline-labeled procollagen after reduction and carboxymethylation with iodoacetic acid. Chromatography was performed in 2 *M* urea–0.025 *M* Tris-HCl (pH 8.5) containing 0.1% 2-mercaptoethanol and protease inhibitors as described in the Experimental Section. Insets adjacent to each radioactivity peak show dodecyl sulfate acrylamide gel electrophoresis patterns of the material eluting in that peak. Gels were stained with Coomassie Blue.

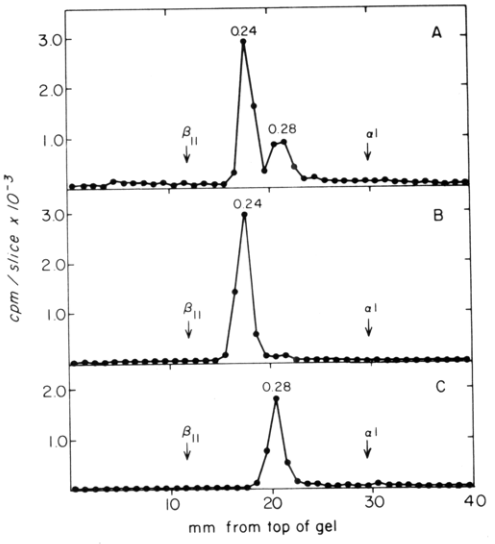

FIGURE 2: Dodecyl sulfate acrylamide gel electrophoresis patterns of (A) the reduced and carboxymethylated [^{3}H]proline-labeled procollagen applied to DEAE-cellulose; (B) the material eluting in the second peak in Figure 1; and (C) the material eluting in the first peak in Figure 1. Arrows mark the positions of dansylated collagen components used as internal standards. R_f's were calculated relative to the migration of dansylated α1-CB7.

a 2:1 ratio (Figure 2A). The material eluting in the second peak migrated as proα1 (Figure 2B) and that in the first peak migrated as proα2 (Figure 2C). The single radioactivity peaks shown in Figure 2B and C correspond in position of migration to the major stained bands (Figure 1, insets). Similar results were obtained when [^{3}H]tyrosine or [^{3}H]tryptophan were used as labels, indicating the presence of these amino acids in each proα chain. In all cases the yield of radioactivity from the DEAE-cellulose column was at least 65%.

Stained dodecyl sulfate gels of the material eluting at the front of the column demonstrated the presence of α1 and α2 (data not shown). Only slight traces of α1 and α2 can be seen on the stained gels in Figure 1. Hence, this chromatographic method not only separated proα1 from proα2, but also separated these chains from the bulk of the contaminating α chains of collagen.

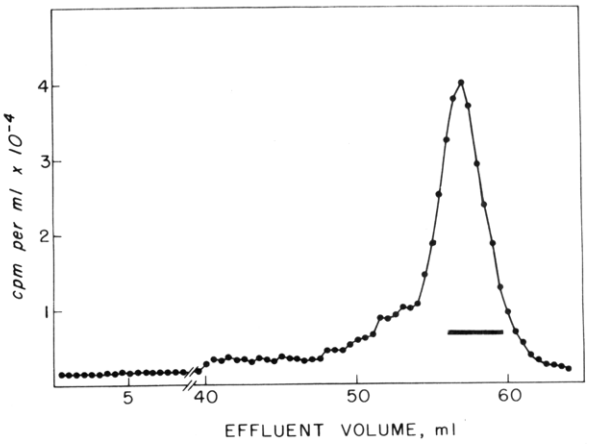

FIGURE 3: Preparative dodecyl sulfate acrylamide gel electrophoresis of [^{3}H]proline-labeled proα1 isolated by DEAE-cellulose chromatography in Figure 1. The bar indicates fractions which were pooled for subsequent analysis.

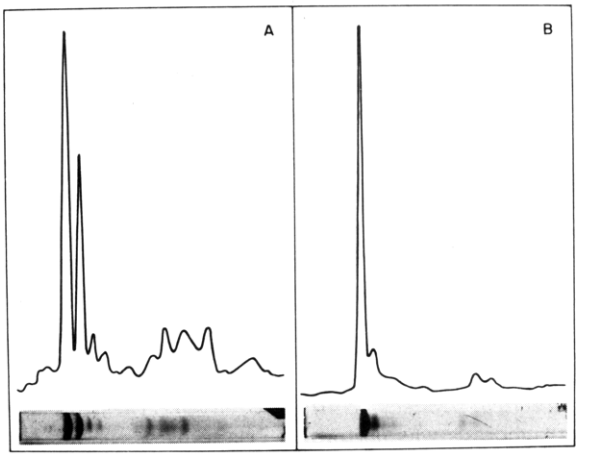

FIGURE 4: Dodecyl sulfate acrylamide gel electrophoresis of proα1 before (A) and after (B) preparative dodecyl sulfate gel electrophoresis. The gels were stained with Coomassie Blue and densitometric tracings were obtained at 565 nm. In each case, the horizontal axis of the densitometric scan is slightly longer than the photograph of the stained gel, accounting for the slight deviation to the right of the trace relative to the gel.

The proα1 chain isolated by DEAE-cellulose chromatography was impure as seen in the stained dodecyl sulfate gel in Figure 1 (right inset). However, the major high molecular weight contaminant was sensitive to bacterial collagenase, suggesting its identity with an altered proα chain which may correspond to one of the recently identified intermediates in the conversion process (Davidson and Bornstein, 1975).

In order to achieve further purification, the proα1 preparation obtained by DEAE-cellulose chromatography was subjected to preparative dodecyl sulfate acrylamide gel electrophoresis (Figure 3). Fractions indicated by the bar were pooled and analyzed by analytical dodecyl sulfate gel electrophoresis. A comparison of the stained gel and densitometric trace of the sample prior to (Figure 4A) and after (Figure 4B) preparative gel electrophoresis indicated that substantial purification was achieved by this method.

Amino acid analyses (Table I) were performed on proα1 isolated by preparative gel electrophoresis. The values given represent the average of six determinations performed on material obtained from three different preparative gels. The

Table I: Amino Acid Composition of Chick Bone Proα1.[a]

	Proα1 Residues per 1000 (±SE)[b]	α1 Residues per 1000[c]	Proα1 Residues per 1540 Res.	Proα1 Calcd[d]
4-Hydroxyproline	67.9 (3.1)	98	105	100
Aspartic acid	61.4 (0.6)	43	95	110
Threonine	31.1 (0.7)	19	48	55
Serine	44.1 (2.6)	32	68	85
Glutamic acid	98.2 (1.1)	81	151	151
Proline	99.6 (1.7)	115	153	145
Glycine	274 (2.2)	333	422	396
Alanine	107 (0.5)	130	165	163
Cystine/2	8.9[e] (0.2)	0	14	17
Valine	24.9 (0.4)	15	38	39
Methionine	10.5 (0.4)	9	16	13
Isoleucine	21.8 (0.9)	7	34	31
Leucine	33.8 (1.2)	19	52	52
Tyrosine	10.0 (0.3)	2	15	17
Phenylalanine	16.9 (0.4)	11	26	25
Hydroxylysine	5.6 (0.6)	6.5	8.6	7.6
Histidine	7.2 (1.3)	3	11	13
Lysine	31.6 (0.8)	26	49	55
Tryptophan	N.D.[f]	0	N.D.	N.D.
Arginine	45.3 (0.6)	47	70	68

[a] Values are the averages of six determinations and are uncorrected for hydrolytic losses. [b] SE = standard error of the mean. [c] From von der Mark and Bornstein (1973). [d] Calculated assuming 200 residues at the NH_2-terminus (von der Mark and Bornstein, 1973), 1000 residues for the α1 chain, and 340 residues at the COOH-terminus (Murphy et al., 1975). [e] Determined as carboxymethylcysteine. [f] N.D. = not determined.

latter were in turn derived from two independent extractions of procollagen. The amino acid composition of proα1 is consistent with a precursor chain comprised of an α1 chain plus additional regions which differ markedly in their amino acid composition. The four amino acid residues which are most characteristic of the α1 chain (hydroxyproline, proline, glycine, and hydroxylysine) were all found in diminished amounts when the compositions of proα1 and α1 were compared (Table I, columns 2 and 3). The analyses confirmed the presence of cystine which is not found in the α1 chain. Also, there was a severalfold increase in the amount of certain amino acids (isoleucine, tyrosine, and histidine) which are relatively deficient in the α1 chain. Although the tryptophan content was not determined in this study, radiochemical labeling has clearly indicated the existence of this amino acid in the proα1 chain (von der Mark and Bornstein, 1973; Murphy et al., 1975).

Discussion

It has been possible for some time to separate the proα1 and proα2 chains of neutral pH extracted procollagen by analytical dodecyl sulfate gel electrophoresis (Monson and Bornstein, 1973). However, the development of a preparative method for the separation of intact proα1 and proα2 chains has proven to be a difficult task. We report here a successful chromatographic method for the separation of these chains from each other and from contaminating α chains (Figures 1 and 2) and a substantial purification of the proα1 chain by preparative dodecyl sulfate gel electrophoresis (Figures 3 and 4). These methods should prove to be useful for further structural studies of these polypeptides.

Development of these methods was hampered by the tendency of the reduced and alkylated proα chains to bind irreversibly to a number of surfaces even in the presence of denaturing agents and by their susceptibility to proteolytic attack. In this work, the solubility of the proα1 chain was enhanced by carboxymethylation with iodoacetic acid and the use of neutral or basic pH buffers, and proteolysis was minimized by addition of protease inhibitors to solutions whenever possible. Precipitation with Cl_3CCOOH in the initial stages of purification served as a convenient means of concentrating protein samples and may have contributed to the inactivation, through denaturation, of contaminating proteolytic activities.

Despite these precautions, proteolytic alteration remained a problem in purification of proα1 and contributed to difficulties in the isolation of the proα2 chain, which appears unusually susceptible. In some instances samples of proα1 purified by preparative gel electrophoresis were essentially homogeneous when initially tested, but displayed heterogeneity in subsequent analysis on dodecyl sulfate gels. Thus, not all the samples analyzed for amino acid composition in this work were stable by this criterion. In spite of this, the analyses were reproducible as demonstrated by the standard errors presented in Table I.

According to our current model, chick bone procollagen contains nontriple helical "noncollagenous" peptide extensions at the NH_2- and COOH-termini of the molecule (Tanzer et al., 1974; Byers et al., 1975 Murphy et al., 1975). We have recently concluded that acid-extracted procollagen represents a truncated precursor which lacks a COOH-terminal disulfide-bonded domain (Byers et al., 1975). In all likelihood, the nontriple helical sequence of acid-extracted proα1 previously analyzed (von der Mark and Bornstein, 1973) contains the major portion, if not all, of the NH_2-terminal extension present in the proα1 chain of the precursor isolated here. The majority of the COOH-terminal extension is released during the normal conversion of procollagen and appears as disulfide-bonded fragment which has been purified from the medium of cultured bone and its amino acid composition determined (Murphy et al., 1975).

The composition of a proα1 chain of 1540 residues (Table I, column 4, assuming an α1 chain of 1000 residues, an NH_2-terminal extension of 200 residues, and a COOH-terminal extension of 340 residues) is consistent with the proposed structure of procollagen. This composition agrees quite well with the composition (Table I, column 5) arrived at independently by summing the compositions of α1, the NH_2-terminal extension in the acid-extracted proα1 (von der Mark and Bornstein, 1973), and the average composition of the COOH-terminal extension in procollagen (Murphy et al., 1975). The last composition represents an estimate since the fragment analyzed contained sequences from both proα1 and proα2 chains. The major discrepancies in columns 4 and 5, Table I, are in serine and aspartic acid. We have previously encountered variability in determination of serine content (Bornstein et al., 1972; von der Mark and Bornstein, 1973); conceivably, variable contamination of the NH_2-terminal extension by a serine and aspartic acid rich protein similar to that found in dentin (Veis and Perry, 1967; Butler et al., 1972) may have led to an overestimation of the content of these amino acids in the earlier work.

The estimated size of a proα chain of 1540 residues, molecular weight approximately 150,000, is dependent on the extent to which the intactness of the precursor was preserved during the extraction and subsequent purification of the protein. The proα chain identified in this study is simi-

lar in molecular weight to the θ chain identified by Church et al. (1974) and considered by those authors to be a fragment of a higher molecular weight chain which serves as a precursor to all three proα chains (Church et al., 1971; Bankowski and Mitchell, 1973; Park et al., 1975). Neither these nor other studies which include pulse chase experiments and an extensive investigation of the procollagen to collagen conversion (Davidson and Bornstein, 1975) have demonstrated the existence of nonreducible chains higher in molecular weight than the proα1 chain characterized in this work. Higher molecular weight precursors may conceivably exist, however, and may have only a short metabolic half-life or may be unusually susceptible to proteolytic attack.

Acknowledgments

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