Collagen Heterogeneity

HIGH RESOLUTION SEPARATION OF NATIVE [α1(I)]2α2 AND [α1(II)]3 AND THEIR COMPONENT α CHAINS*

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SUMMARY

The two collagens of cartilage differing in primary structure, extracted from lathyritic chick xiphoid processes, can be separated from each other in their native forms by differential salt precipitation. One form, [α1(II)]α2, present in smaller amount, is less soluble and is precipitated by 2.2 M NaCl at neutral pH; the other form, [α1(I)]α3, which remains in solution, can be precipitated by dialysis at very low ionic strength.

The two different α1 chains, α1(I) and α1(II) which chromatograph close to one another on CM-cellulose, can be completely separated under mildly alkaline conditions on an anion exchange resin, DEAE-cellulose, by stepwise elution with buffers of decreasing pH. This chromatographic technique for collagen chain separation has revealed microheterogeneity of both types of α1 chains by separating a number of components displaying only small differences in amino acid content, principally in the hydroxylysine moiety.

In a given vertebrate species it is now apparent that different collagens exist in different tissues (1, 2). To date, the collagen in bone and adult skin (3, 4), in embryonic skin (2), in cartilage (5, 6), and in basement membranes (7) have been shown to differ in amino acid sequence and α chain distribution. In addition, there are microheterogeneities based on variation in degree of hydroxylation of proline and lysine (8-11), glycosylation of hydroxylysine (12, 13), aldehyde content (14) and size of Nlinked possibly COOH terminal modified peptides (15, 16). Recent studies of native calf skin collagen on hydroxyapatite columns have revealed chromatographic heterogeneity, but further characterization of these fractions was not reported (17).

In cartilage, the predominant molecular species consists of three identical α1 type II chains, or [α1(II)]α3, and a lesser amount of collagen similar to that found in skin and bone composed of two α1 type I chains and one α2 or [α1(I)]α2. This latter molecule is presumably derived from the perichondrium and is readily removed in the early tissue extracts; subsequent extraction of mineed cartilaginous tissue yields [α1(II)]α3 almost exclusively (5, 6). The two different α1 chains cannot be separated by the classical CM-cellulose chromatography (18).

We now report the separation of native [α1(I)]α2 from native [α1(II)]2 by fractional salt precipitation and a high resolution chromatographic separation of α1 type I and α1 type II chains (designated α1(I) and α1(II), respectively) on DEAE-cellulose. This latter method also fractionates α1 chains differing only slightly in their amino acid composition.

MATERIALS AND METHODS

Collagen Purification and Separation by Salt Fractionation—White Leghorn chickens, 1 week of age, were made lathyritic by addition of β-amino-propionitrile (BP) to their drinking water at a concentration of 0.03% for 2 weeks. Two weeks of exposure to the drug, the animals were killed and the xiphoid cartilage was dissected free from surrounding tissues. The tissues were diced into 1-mm cubes and extracted overnight at 4°C with 4.4 M ionic strength phosphate buffer, pH 7.6, with gentle stirring. Three separate extractions were performed, and all subsequent manipulations were done at or near 4°C.

The pooled extracts were clarified by centrifugation, and the first fractional precipitation of collagen was accomplished by slowly adding an equal volume of cold 4.4 M NaCl and allowing the thoroughly mixed solution to sit overnight. The precipitate was collected by centrifugation at 5000 × g for 30 min and redissolved by stirring in phosphate buffer. The supernatant was dialyzed against 0.01 M Na2HPO4 with several changes over a 24-hour period, and the precipitate was collected by centrifugation and redissolved in phosphate buffer. By this two-step fractionation, an estimated 80-90% of the [α1(I)]α2 precipitated at 2.2 M NaCl while the [α1(II)]α3 remained soluble. The precipitation of the more soluble [α1(II)]α3 can be accomplished by either further addition of solid NaCl to a final concentration of 4.4 M or by dialysis against low ionic strength buffer, 0.01 M Na2HPO4. The latter method allowed quantitative recovery, hence, it was preferred. To assure complete separation of [α1(I)]α2 and [α1(II)]α3, the resolubilized precipitates from the

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first fractionation were refractionated twice again under the same conditions. Each of these fractions was then precipitated from 0.4 ionic strength phosphate solution by rapid addition of cold 100% ethanol to a final concentration of 14%. After sitting overnight, the precipitates were collected by centrifugation and dissolved in 0.1 M acetic acid. The protein was then reprecipitated by adding 4.4 M NaCl to a final concentration of 2.2 M. Both collagen molecules precipitate at this salt concentration at the acid pH. The precipitates were collected, dissolved in and dialyzed against 0.1% acetic acid to remove salt, lyophilized, and stored at -40°.

Cartilage and bone collagen from lathyritic chickens, which had also been rendered rachitic by feeding a vitamin D- and phosphate-deficient diet (11), were prepared from the ends of the long bones. This cartilaginous and osseous tissue was pulverized after freezing in liquid nitrogen and extracted once with 0.4 ionic strength phosphate, pH 7.6. The solubilized collagen was precipitated by adding solid NaCl to a final concentration of 3.1 M. This material was not fractionally precipitated, but was subsequently purified by neutral precipitations from solution of 0.4 ionic strength phosphate with 14% ethanol and dialysis against 0.01 M Na₂HPO₄ followed by acid precipitation in 2.2 M NaCl as described above. The purified mixture of cartilage and bone collagens was desalted, lyophilized, and stored at -40°. This material was used to develop the conditions of separation of α₁(Ⅰ), α₁(Ⅱ), and α₂ by DEAE-cellulose chromatography.

Skin collagen from lathyritic chickens was prepared and purified as described above for rachitic bone and cartilage collagen.

CM-cellulose Chromatography—Chromatography of the purified heat-denatured collagens on CM-cellulose was performed using jacketed columns (0.9 × 4.5 cm) equilibrated at 41° with a pH 4.85 acetic buffer containing 0.06 M sodium acetate and 0.038 M acetic acid. The retained collagen chains were eluted with a linear gradient of NaCl from 0 to 0.1 M over a total volume of 200 ml (18). The effluent of the columns was monitored continuously at 228 to 230 nm in a Gilford spectrophotometer equipped with a flow cell.

DEAE-cellulose Chromatography—Samples of desalted lyophilized α chains obtained from CM-cellulose chromatography were solubilized in 0.004 M Tris-HCl, pH 9.7, and applied to a jacketed column (0.9 × 4.5 cm) containing DEAE-cellulose (Whatman DE 52) which had been equilibrated with the same buffer. The α chains were then eluted by a stepwise pH gradient consisting of 0.004 M Tris-HCl buffers of decreasing pH. The column temperature was maintained at 41° and the flow rate was 40 to 50 ml per hour. The effluent from the column was monitored in a spectrophotometer as described above. All buffers were prepared from Tris base by dissolving 1 mole (121 g) in approximately 850 to 900 ml of distilled water, adjusting the pH with HCl and bringing the final volume to 1 liter. The actual pH of the buffer solution under the conditions of chromatography, i.e. 0.004 M at 41°, was substantially lower (usually 0.5 to 0.9 unit) than that of the 1.0 M stock. Following use the DEAE-cellulose was regenerated in the column by washing with 0.5 M Tris-HCl, pH 5.0, containing 0.5 M NaCl followed by 0.1 M Tris-HCl, pH 9.70. After the effluent became alkaline, the resin was re-equilibrated with 0.004 M Tris-HCl, pH 9.70, and reused. The performance of the DEAE-cellulose did not significantly change despite repeated use.

Disc Gel Electrophoresis—Polyacrylamide disc gel electrophoresis was done according to the method of Stark and Kühn (10) and the Amido black-stained gels were scanned photometrically.

Amino Acid Analysis—Amino acid analyses were performed using the single column method on an automatic instrument (Beckman 120C) modified for high speed analyses (20). Samples were hydrolyzed in constant boiling HCl at 108° for 24 hours in a tube sealed under nitrogen. Correction factors for the labile amino acids (threonine, serine, methionine, and tyrosine) and for the incomplete release of valine were applied as previously determined (21).

Electron Microscopy—Segment long spacing crystallites of the salt-fractionated collagens were prepared by dialysis of collagen solutions, in 0.05 M acetic acid, pH 3.5, against 0.4% salt-free ATP. The resultant suspension was applied to grids, stained with uranyl acetate and examined in an RCA EMU 3G electron microscope.

RESULTS

Fractional Salt Precipitation of Lathyritic Xiphoid Cartilage Extracts—The first neutral extract of lathyritic xiphoid cartilage has previously been shown to consist of a mixture of [α₁(Ⅰ)α₂] and [α₁(Ⅱ)α₂]. The total amount of collagen extracted and the proportion of the two molecules obtained is strongly dependent on the tissue preparation. Extracts of crudely diced cartilage, as used in the present study, contain much less total collagen, but a more equal mixture of the two collagens, whereas pulverized cartilage extracts contain more collagen with a higher proportion of [α₁(Ⅰ)]. We deliberately employed dicing to enhance the relative amount of [α₁(Ⅰ)α₂] in the first extract (Table I) and the total yields were therefore less than have been previously reported (5, 6). When the first extract from minced cartilage was subjected to fractional salt precipitation, the 2 collagen molecules were effectively separated as revealed by the following criteria: (a) chromatographic profile of the heat-denatured collagen on CM-cellulose; (b) amino acid composition of both the native collagen and the α chains obtained by CM-cellulose chromatography; and (c) electron microscopic appearance of the segment long spacing crystallites. By all of these criteria, the material precipitating at 2.2 M NaCl is [α₁(Ⅰ)α₂], whereas the more soluble material which was precipitated by low ionic strength dialysis was [α₁(Ⅱ)].

Amino acid analyses of the native collagens obtained by salt fractionation (Table II) are consistent with the designation of

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<th>Salt fraction of lathyritic xiphoid cartilage</th>
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<td>2.2 M NaCl precipitate</td>
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1 Micrometer crystals of collagen molecules aligned in parallel and close register are precipitated from acid solution with ATP. The highly reproducible detailed band pattern of these structures brought out by heavy metal staining and seen electron microscopically represents the linear distribution of charged groups.
the 2.2 m NaCl precipitable fraction as \([a_1(1)]a2\) and the 2.2 m NaCl soluble fraction as \([a_1(II)]a2\).

Chromatograms on CM-cellulose of the two fractions obtained from the first extract are illustrated in Fig. 1. The less soluble collagen contained both \(a1\) and \(a2\) chains in a ratio of 2.0 to 1. Amino acid analyses of these \(\alpha\) chains (Table II) clearly identified them as \(a1(I)\) and \(a2\). The more soluble collagen contained only \(a1\) chains which were identified as \(a1(II)\) by amino acid analyses (Table II). Since both chromatograms were done under identical conditions, the superimposed tracings shown in Fig. 1 illustrate the relative elution positions of the two types of \(a1\) chains on CM-cellulose. These results confirm our earlier observations that \(a1(II)\) is more acidic than \(a1(I)\) (6), an observation also confirmed more clearly by the anion exchange chromatography described below.

Segment long spacing crystallites of \([a1(II)]a2\), are different from those of skin and bone collagen in the relative staining densities of particular groups of bands (6); using this, we could show that the 2.2 m NaCl precipitate behaved like the collagen of skin and bone, and segment long spacing crystallites of the more soluble collagen resembled \([a1(II)]a2\).

**DEAE-cellulose Chromatography.**—Approximately 65 different chromatographic conditions were tested in an effort to separate \(a1(I)\), \(a1(II)\), and \(a2\) on DEAE-cellulose. All of these modifications were based on the observation that \(a1(II)\) would not be retained by the resin in low ionic strength Tris-HCl buffer (0.008 M) prepared from 1.0 M stock, pH 8.5, whereas \(a1(I)\) would, and further, that both types of \(a1\) chains would be retained at low ionic strength and higher pH (9.0 to 9.7). The desired conditions were, therefore, those in which both types of \(a1\) chains would bind to the column and then be sequentially and separately eluted. Four different types of elutions were attempted: (a) linear salt gradients of NaCl at constant pH; (b) linear salt gradients of NaCl superimposed on a decreasing pH gradient; (c) decreasing pH gradient with either constant or unequal strengths of the two buffers, and (d) stepwise pH gradients at constant total buffer concentration. Results with the first three types of gradients were nearly always unsatisfactory because the salt caused large absorbance peaks which often obscured the protein absorbance, and, more important, separation of the different \(\alpha\) chains was incomplete. On the other hand, stepwise elution with buffers of constant total buffer concentration but different pH owing to an increased concentration of HCl will completely separate \(a1(I)\) and \(a1(II)\).

The chromatographic technique is reproducible with different batches of resin and buffers. Although the buffer pH decreases significantly with dilution and heating, this has not been a problem as long as the pH of the 1.0 M Tris-HCl stock buffer is measured at room temperature.

Although most \(a1\) chains will be retained by the DEAE-cellulose at pH 9.0, 0.004 M, a higher pH of 9.7 was chosen as the starting buffer. This choice was dictated by the fact that the solution of denatured collagen exerts a considerable Donnan effect when dialyzed against the starting buffer. One consequence of this is that the pH in the sample was usually 0.5 unit lower than that in the dialysate. This effect has been noticed even at pH values closer to the pK, of Tris where buffering capacity is maximal. The lower pH of the sample when loaded, however, usually does not affect the results and is probably insignificant because of the large buffering capacity of the resin compared to that of the sample.

Chromatography on CM-cellulose of the mixture of bone and cartilage collagen purified from the ends of the rachitic, lathyritic chick long bones resolved only two peaks, the first containing both types of \(a1\) and the second \(a2\). When this isolated peak of \(a1\) chains was applied to DEAE-cellulose, two widely separated fractions were eluted by the stepwise pH changes (Fig. 2). No discernible artifact peaks at the void volume of the column were caused by the buffer changes. The first peak to elute with 0.004 M Tris-HCl, pH 8.35, was slightly heterogeneous and on electrophoresis in polyacrylamide gels the two portions of this peak migrated as \(a1\) chains with very little \(\beta\) components present (Fig. 3). Amino acid analysis of these two portions of the peak indicated that both were \(a1(I)\) chains, but the material in Peak 2
ELUTION VOLUME, mL

FIG. 2. DEAE-cellulose chromatogram of the α1 chains obtained by CM-cellulose chromatography of collagen from the ends of chick long bones. The different α1 chains, α1(I) and α1(II), have been separated and identified by amino acid analysis. The heterogeneity in α1(I) is apparently due to differences in degree of lysyl hydroxylation. The pH indicated for the buffer is that of the 1.0 M Tris-HCl stock solution at room temperature. The actual pH of the 0.004 M Tris-HCl buffer at 41° is from 0.5 to 0.9 unit lower.

Fig. 3. Densitometric tracings of polyacrylamide gels following electrophoresis of the heterogeneous α1(I) region of Fig. 2. Both Peaks 1 and 2 are predominantly α chains.

in Fig. 4 suggested that DEAE-cellulose might separate α chains of common primary structure on the basis of the microheterogeneities described in the introduction. To test this possibility, a mixture of hydroxylsine-rich α1(I) from rachitic chick bone (11) and hydroxylsine poor α1(II) from adult lathyritic chick skin was examined; both of the α1(I) chains had been obtained by prior CM-cellulose chromatography. The data shown in Fig. 5 indicate that these α1(I) chains can be separated into multiple components by stepwise pH elution. The order of elution, except for Peak 5, was coincident with the degree of lysyl hydroxylation; Peak 1, 10.8%; Peak 2, 13.8%; Peak 3, 18.8%; Peak 4, 23.7%; Peak 5, 18.6%; Peak 6, 30.0%. Experiments are currently in progress to determine whether this also represents a progression in the degree of glycosylation (12, 13). Peaks 3 and 5, both minor components, contained 1 to 2 residues of half-cystine per 1000 residues. All peaks contained 333 residues of glycine per 1000 except Peak 5 which contained 316 glycine, 80 4-hydroxyproline, 51 serine, 93 glutamic acid, 1.7 half-cystine, 4.7 tyrosine, and 6.4 histidine residues per 1000.

The recovery of α chains from the DEAE-cellulose columns was similar to that obtained from the CM-cellulose columns as
measured by planimetry of the eluted peaks (3). Addition of 2 M urea to the DEAE buffers reduced the resolution and did not significantly improve the yields. Rechromatography of α chains separated on DEAE-cellulose showed reproducible elution behavior.

Chromatography of a denatured mixture of collagen directly applied to DEAE-cellulose usually separated the three distinct α chains, α2, α1(1), and α1(11). However, initial retention of α2 chains to the resin was unreliable especially with material from older animals. When retained, α2 was eluted with 0.004 M Tris-Cl, pH 9.0 to 8.8, although it may elute in the buffers at lower pH near that used for α1(1). The easy separation of α2 from α1 on CM-cellulose prior to use of DEAE-cellulose readily eliminates this problem.

**Discussion**

Although lathyritic chick xiphoid cartilage contains two distinct collagens [α1(1)α2 and [α1(11)]2] which are quite similar in some of their solubility characteristics, it is clear from the present work that they differ sufficiently to be separated by salting out with sodium chloride from neutral solution. The reason for this solubility difference may reside in the carbohydrate side chains. Each [α1(11)]2 molecule contains approximately 10 times as much covalently bound hexose as does skin or bone collagen (5, 6) and carbohydrate side chains are known to generally increase protein solubility (22). The difference in α chain distribution and primary structure of the two cartilage collagens also may be responsible for some of the solubility differences, but these factors appear to have little influence on other physical-chemical properties such as intrinsic viscosity, optical rotatory dispersion, or molecular weight determined by sedimentation equilibrium in which [α1(1)α2 and [α1(11)]2] are nearly identical.

Differential solubility may be an effective tool in separating the other distinct collagen molecules which have been found in the higher vertebrates in embryonic skin (2) and basement membranes (7). In addition, these fractionation methods may be useful in isolating the various precursor forms of collagen α chains or procollagen which have unusual solubility properties (23-25). By differential solubility, we have isolated yet another form of collagen from embryonic chick skin containing α1 chains of unusual amino acid composition (26). This material may be related to the peptides obtained by cyanogen bromide treatment of human fetal skin which Miller et al. (2) postulate derives from an α1(III), or possibly to the precursor forms of the α1(1) and α2 (24, 25).

The separation of collagen α chains by DEAE-cellulose chromatography occurs not only because of charge differences in the polypeptide chains derived from primary structural differences, but presumably also because of charge differences brought about by post-translational enzymatic modifications of identical chains. In the present study, we have intentionally employed α chains from skin, bone, and cartilage from different sources, e.g. xiphoid cartilage and long bone cartilage and from animals in altered biological states, e.g. lathyritic and rachitic in order to determine the extent to which DEAE-cellulose chromatography can be applied to studies of collagen heterogeneity. The separation of the two α chains, α1(1) and α1(11), was always complete regardless of the source of the materials. The fractionation achieved with α1 chains of common primary structure indicates that significant microheterogeneity exists,

and the initial observations on α1(1) suggest that variation in hydroxylation of lysyl residues may be the cause of the altered elution behavior. This may occur through changes in net charge on the α chain because the pKα of the ε-amino group of hydroxylysine (9.67) is lower than that of lysine (10.53) (27), or perhaps because of changes brought about by glycosylation of these hydroxylysyl residues. In addition, minor components with unusually high numbers of acidic residues such as Peak 5 (Fig. 5) may also separate because of these additional anionic groups.

DEAE-cellulose chromatography cannot as yet be used to identify α chain species by elution position alone since the behavior of α1(11), and α1 chain of basement membranes, the procollagens, and internal organ collagens have not yet been studied. If, however, these prove to be separable by the method described above or modifications of it, a powerful tool will be available for the investigation of heterogeneity in small fragments of tissue by biosynthetic techniques using radioisotopic labels and known carriers.

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**References**

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