

Collagen Synthesis: A Disulfide-Linked Collagen Precursor in Chick Bone*

(calvaria/ultracentrifugation)

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ABSTRACT Chick calvaria labeled *in vitro* contain a triple-stranded collagen precursor that contains two pro $\alpha 1$ and one pro $\alpha 2$ chains. All chains contain cystine and are linked by S-S bonds. Ultracentrifugal analysis was used to measure the relative size of the disulfide-linked units. It is proposed that the S-S links help to determine the correct alignment of the triple-stranded structure.

Recently we reported that cells that synthesize collagen in culture accumulate a disulfide-linked, triple-stranded soluble procollagen (1); metabolic studies (2, 3) have shown this to be a collagen precursor, as was proposed earlier (4, 5). In contrast, collagen precursors from whole tissue have been reported as triple-stranded molecules (6-8) whose component polypeptide strands fully dissociate on denaturation, although these components contain half-cystine residues (8, 9).

We have now found, by rapid extraction of embryonic calvaria bones, a triple-stranded collagen precursor that contains cystine in each chain, requires reduction before strand separation occurs on denaturation, and is therefore presumably linked by disulfide bridges. It contains two pro $\alpha 1$ and one pro $\alpha 2$ collagen chains; we call it pro $\gamma 112$.

MATERIALS AND METHODS

Described methods (1) were used for carboxymethyl cellulose (CMC) chromatography, SHCH₂CH₂OH reduction, and acrylamide-gel electrophoresis, except for modification of technique where noted. To obtain primarily procollagen, we preincubated calvaria from 18-day chick embryos for 1 hr with NH₂CH₂CH₂CN (64 μ g/ml) and ascorbic acid (100 μ g/ml), and then incubated the samples for 18 min at 37° with 28 μ Ci/ml of [5'-³H]proline (Becton-Dickson, 31.5 Ci/mmol) and 35 μ Ci/ml of [³⁵S]cystine (Becton-Dickson, 725 Ci/mmol). To obtain primarily collagen, we continued the incubation for up to 2 hr.

After incubation, calvaria were quickly washed at 0° with H₂O, homogenized in 0.5 M CH₃COOH-0.1% Triton X-100 (Rohm and Haas), and immediately centrifuged (Sorvall SS34 rotor, 15,000 rpm, 30 min); the supernatant was frozen

Abbreviations: CMC, carboxymethyl cellulose; GSSG, oxidized glutathione.

Nomenclature: 'Procollagen' here means material rapidly extracted from calvaria, in contrast to "prolonged extraction procollagen" (6). The component chains of completed collagen triplet molecules are [$\alpha 1$]₂, $\alpha 2$, and of procollagen [(pro $\alpha 1$)₂, pro $\alpha 2$]. Covalently linked pairs of finished chains are called β and triplets, γ , with pro β and pro γ used for corresponding compounds of pro α chains.

* This is paper III of a series. Paper II is ref. 1.

at -16°. Prolonged extraction yielded 10-20% more labeled collagen. For CMC chromatography the acid extract was made 6 M in urea at 70° and heated for 10 min. CH₃COONa was added to 40 mM and pH was adjusted to 4.8.

Native procollagen solutions were treated with 10 mM oxidized glutathione (GSSG) (Cal Biochem) and 10 mM "Diamide" [(diazinedicarboxylic acid)-bis-dimethylamide] (Cyclo Co.) (10) in 0.5 M NaCl-0.1 M Tris buffer (pH 8.2) for 18 hr at 0°. Diamide alone had no effect on the proteins. Immediately before use, concentrated GSSG solutions were filtered by pressure through dialysis tubing to remove proteolytic contaminants.

Ultracentrifugal velocity-band sedimentation analyses were made in 5-20% sucrose gradients in buffers with markers as noted in text, in a Beckman SW56 rotor at 53,000 rpm. A 0.25-ml pad of 60% sucrose at the bottom of each tube assured detection of rapidly sedimenting, aggregated material. CMC chromatography was used to prepare markers of $\alpha 1$, and of $\beta 12$, which was contaminated with small amounts of $\gamma 112$ and $\alpha 2$. These markers had consistent, reproducible sedimentation behavior. Viscosities and densities of the concentrated urea-sucrose solutions were determined and corrections were computed for them throughout each gradient.

RESULTS

Native calvarial procollagen sedimented about 1.15-times faster than collagen; less than 10% of the total sample was in higher aggregates, which would have accumulated at the bottom of the sucrose gradient (Fig. 1). Upon denaturation, procollagen gave the sedimentation pattern of Fig. 2a. Repetition of this experiment after reduction with SHCH₂CH₂OH

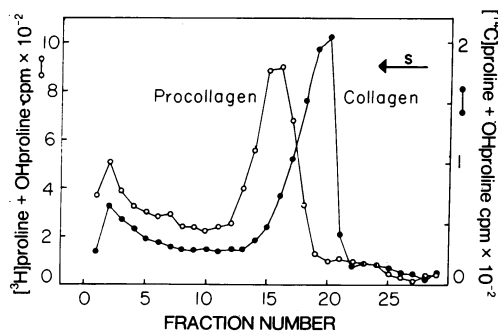


FIG. 1. Velocity sedimentation under NATIVE conditions of extracts of calvaria labeled for 18 min with [³H]proline (O—O) and for 2 hr with [¹⁴C]proline (●—●). Sedimentation from right to left for 15 hr at 7° in 50 mM KCl-10 mM CH₃COOH.

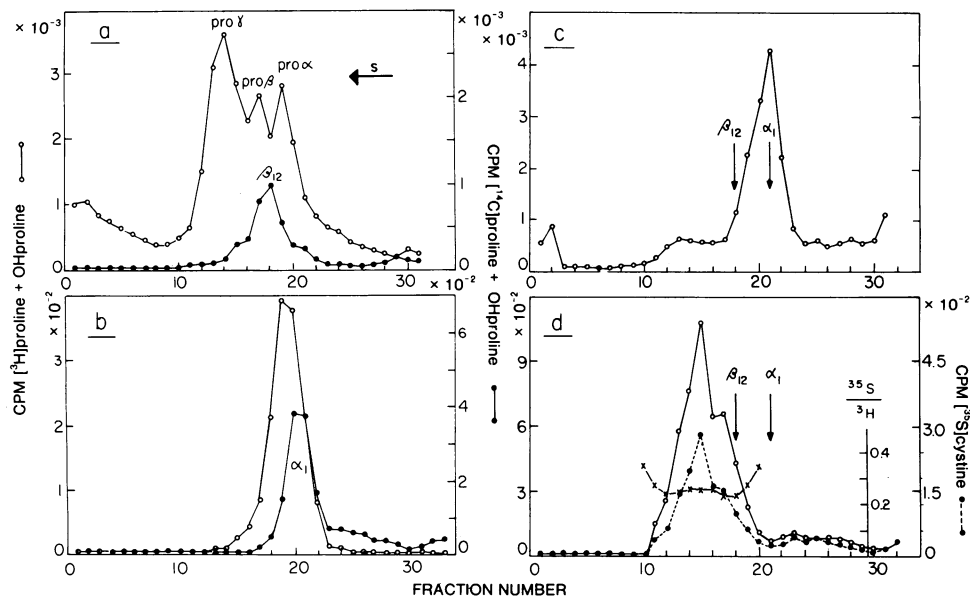


Fig. 2. Velocity sedimentation under DENATURING conditions. (a, b, and c): extracts of calvaria were labeled for 18 min with [^3H]proline (O—O). Then: (a) cosedimented with [^{14}C] β_{12} marker (containing γ_{112} and α_2) (●—●); (b) reduced with 5% $\text{SHCH}_2\text{CH}_2\text{OH}$ in 0.3 M Tris (pH 8.2) at 22° for 18 hr then cosedimented with [^{14}C] α_1 marker (●—●); (c) transferred to nonradioactive medium containing 250 $\mu\text{g}/\text{ml}$ of cycloheximide and incubated for 70 min more. (d) Pooled fractions of peak II of CMC chromatogram shown in Fig. 3. [^3H]Proline (O—O) and [^{35}S]cysteine (●—●) radioactivities are shown together with the ratio ^{35}S cpm/ ^3H cpm (X—X) where this could be determined. Average ratio for fractions 13–16 was 0.26 and for fractions 17–18, 0.23. Sedimentation was from right to left for 18 hr at 20° in 6 M urea–0.1 M NaCl–0.1% Triton X-100–0.04 M CH_3COONa (pH 4.5); in addition b contained 0.1% $\text{SHCH}_2\text{CH}_2\text{OH}$. Arrows indicate position of markers sedimented in separate centrifuge tubes. Denaturation was assured by heating 0.3 ml-samples to 40° for 30 min before layering on top of gradient solutions. Fig. 2a was selected to demonstrate the position of pro β , which is frequently present in amounts smaller than shown.

gave the pattern of Fig. 2b, with a single major peak that sedimented faster than added α_1 marker and with the same velocity as the peak labeled pro α in Fig. 2a. The sedimentation velocity of the peak labeled pro γ Fig. 2a was greater than that of β_{12} , and its velocity relative to the pro α peak was consistent with its being a trimer of three pro α chains. The ratio of the sedimentation velocities of the peaks labeled pro β and pro α in Fig. 2a was the same as that of β_{12} and α_1 , namely 1.33:1 (11). The sedimentation behaviors of pro α chains prepared by rapid and by prolonged extraction methods were similar (not shown).

The precursor nature of the faster sedimenting species was shown by pulse-chase experiments. When protein synthesis was inhibited by addition of 250 $\mu\text{g}/\text{ml}$ of cycloheximide 18 min after start of labeling and incubation was continued for a further 70 min in the absence of [^3H]proline, the procollagen was converted to collagen. This collagen sedimented as α -sized chains upon denaturation *without* reduction (Fig. 2c). Electrophoretic analysis indicated that about 75% of the total material sedimented in Fig. 2c consisted of α_1 and α_2 chains. When 10 mM proline was added in place of cycloheximide, results consistent with these conclusions were obtained.

For determination of the chain composition of pro γ , it was isolated by CMC chromatography and reduced; the pro α chains were identified by electrophoresis. Fig. 3 shows a CMC chromatogram of denatured procollagen labeled with [^3H]proline and [^{35}S]cysteine. Samples from the three peaks (I, II, and III) were reduced with $\text{SHCH}_2\text{CH}_2\text{OH}$ and subjected to electrophoresis. Fig. 4 shows that peak I contained mostly pro α_1 chains and peak III mostly pro α_2 chains, while peak II contained both pro α_1 and pro α_2 . Sedimentation of peak-II material confirmed that it was mostly trimerically linked pro γ with some pro β (Fig. 2d). Detailed analysis of the electrophoretic and sedimentation data shows that the trimer is pro γ_{112} and the dimer is pro β_{12} (Table 1). These data also show clearly that both pro α_1 and pro α_2 chains contain cysteine, but in different amounts. Consistent ^{35}S : ^3H ratios were found for each chain (Fig. 4) independently of CMC chromatographic position of the source material. From these ratios, the ^{35}S : ^3H ratio of pro γ_{112} and pro β_{12}

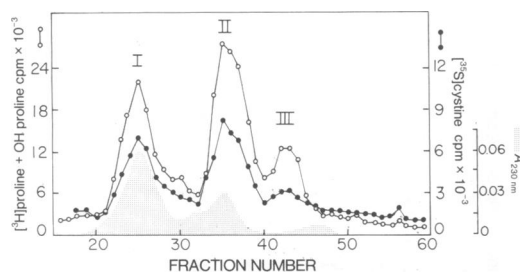


Fig. 3. CMC chromatogram (1) of extract of calvaria labeled for 18 min with [^3H]proline (O—O) and [^{35}S]cysteine (●—●). Shaded area indicates $A_{230\text{nm}}$ of the extract itself, as carrier collagen was not added. Conductivity changed linearly with fraction number, and the conductivities of the fractions in the $A_{230\text{nm}}$ peaks were those at which calvarial α_1 and α_2 chains elute. No significant amounts of radioactive protein eluted earlier than fraction 17.

TABLE 1. Relative ^{35}S content of ^3H -labeled procollagen chains

	Velocity sedimentation analysis (cpm ^{35}S /cpm ^3H)	Electrophoretic* analysis of reduction products (cpm ^{35}S /cpm ^3H)
Observed		
pro $\alpha 1$	0.32 ₀ †	0.32 ₅
pro $\alpha 2$	0.16 ₀ †	0.14 ₆
pro $\beta 12$	0.23 ₀ ‡	—
pro $\gamma 112$	0.25 ₇ §	—
Calculated		
pro $\gamma 112$ ¶	0.26 ₇	0.26 ₅
pro $\beta 12$	0.24 ₀	0.23 ₅

The subscript digit is as experimentally determined, but the authors place reliance only on the first two digits.

* Data from Fig. 4.

† From separate sedimentation analyses of material from peaks I and II of CMC chromatogram of Fig. 3.

‡ From Fig. 2d; average of fractions 17–18 (0.230, 0.231).

§ From Fig. 2d; average of fractions 13–16 (0.248, 0.269, 0.258, 0.253).

¶ $(^{35}\text{S}/^3\text{H} \text{ of pro } \gamma 112) = [2(^{35}\text{S}/^3\text{H} \text{ of pro } \alpha 1) + (^{35}\text{S}/^3\text{H} \text{ of pro } \alpha 2)] \div 3$.

|| $(^{35}\text{S}/^3\text{H} \text{ of pro } \beta 12) = [(^{35}\text{S}/^3\text{H} \text{ of pro } \alpha 1) + (^{35}\text{S}/^3\text{H} \text{ of pro } \alpha 2)] \div 2$.

were calculated and agreed well with the sedimentation results of Fig. 2d. We conclude that electrophoresis or sedimentation separated collagen precursor chains from ^{35}S -cysteine-containing impurities.

The ratio of ^3H proline in peaks I and III of Fig. 3 is 2:1. This value implies that there were several species of procollagen molecules, all of the form $[(\text{pro } \alpha 1)_2 (\text{pro } \alpha 2)]$, but S-S linked to different extent. Treatment of native procollagen with GSSG and diamide increased the proportion of disulfide-linked pro $\gamma 112$ 1.6 fold, as determined by ultracentrifugation or CMC chromatography. The latter showed a corresponding increase in peak II (pro $\gamma 112$) and decreases in peaks I (pro $\alpha 1$) and III (pro $\alpha 2$). After GSSG oxidation, the peak II material sedimented entirely as trimerically linked chains (Fig. 5), and after $\text{SHCH}_2\text{CH}_2\text{OH}$ reduction it was separated into pro $\alpha 1$ and pro $\alpha 2$. Native procollagen that had been treated with GSSG and diamide maintained the collagen portion in the native collagen configuration; limited pepsin digestion followed by denaturation gave $\alpha 1$ and $\alpha 2$ chains in good yield and ratio 2:1, as determined electrophoretically.

DISCUSSION

The results demonstrate that a triple-stranded disulfide-linked procollagen, consisting of two pro $\alpha 1$ and one pro $\alpha 2$ chains, can be isolated from calvaria incubated in the presence of $\text{NH}_2\text{CH}_2\text{CH}_2\text{CN}$, which prevents formation of other collagen crosslinks. Clearly all precursor strands have the potential for interstrand disulfide bridging. We have observed both increase and decrease in S-S linkage after materials were handled at neutral pH for as little as 1 hr. It is important to minimize alterations in disulfide bridging, and we therefore used acid solutions, which suppress thiol ionization. A definite biosynthetic significance for the observed S-S linkage is indicated by preliminary time sequence studies (not shown). These show that the proportion of precursor that is S-S

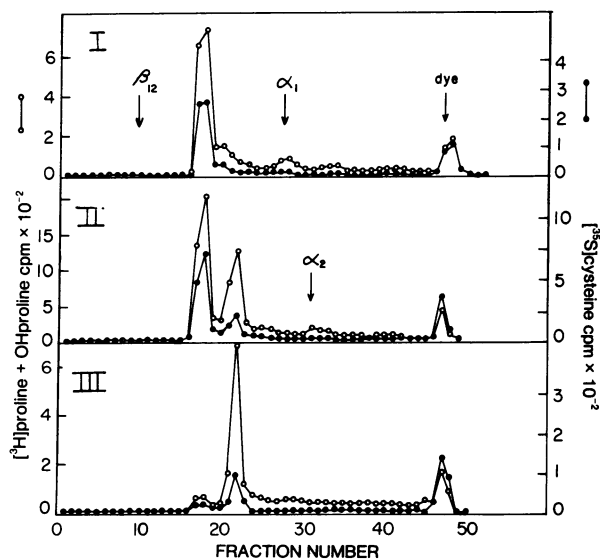


FIG. 4. Electrophoretograms of peaks I, II, and III from CMC chromatogram of Fig. 3 after $\text{SHCH}_2\text{CH}_2\text{OH}$ reduction. ^3H Proline (O—O); ^{35}S cysteine (●—●). Positions of $\beta 12$, $\alpha 1$, and $\alpha 2$ were determined both by relative mobility and by markers run independently in parallel. 18-cm, 5% acrylamide gels containing 0.5% Na dodecyl sulfate, 0.5 M urea, and 0.1% $\text{SHCH}_2\text{CH}_2\text{OH}$.

crosslinked increases with time until cleavage to final collagen occurs.

Our CMC chromatograms (Fig. 3) failed to show significant elution of material before peak I. Correspondingly, we find calvarial pro $\alpha 1$ cochromatographs with $\alpha 1$, as we had found earlier for pro $\alpha 1$ from fibroblasts (1). We conclude that calvarial pro $\alpha 1$, which has been reported (6) to elute from CMC at lower ionic strength, is a component of a procollagen that has been partly degraded during prolonged acid extraction. While this manuscript was in preparation Monson and Bornstein (12) briefly reported similar findings, using extraction at neutral pH together with inhibitors of proteolytic enzymes. Our results show that care must be exercised in using CMC chromatography for characterization of pro-

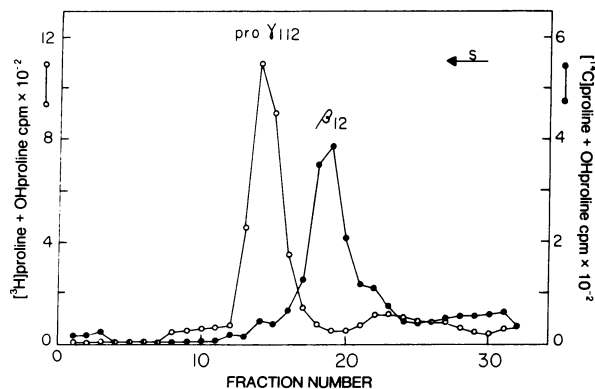


FIG. 5. Effect of treatment with GSSG and diamide on a native extract of calvaria labeled for 18 min with ^3H proline. After denaturation and CMC chromatography, peak II material was isolated; this is the sedimentation pattern under the same DENATURING conditions used in Fig. 2d. ^3H Proline (O—O); cosedimented ^{14}C $\beta 12$ marker (●—●).

collagen and collagen components: pro $\alpha 1$ cochromatographs with $\alpha 1$ and pro $\gamma 112$ with $\alpha 2$, small amounts of pro $\beta 12$ appear as a shoulder of the pro $\gamma 112$ peak, and probably pro $\beta 11$ forms a shoulder to the pro $\alpha 1$ peak. The pro β components may represent both biosynthetic intermediates and material damaged during extraction.

Ultracentrifugation has distinct advantages for discrimination of molecular species in the size range of γ chains (13). Others (e.g., refs. 2 and 5) have used molecular-sieve chromatography and electrophoresis in various denaturant solutions for identification of disulfide-linked material, but resolution and assignment become uncertain for species greater than β marker chains. In contrast, ultracentrifugal analysis becomes easier as sedimentation velocity increases with molecular size, e.g., our data indicate that pro $\gamma 112$ trimers are not significantly further S-S linked into higher aggregates.

The mechanism of selection and assembly of two pro $\alpha 1$ and one pro $\alpha 2$ chains into a triple-stranded molecule is likely to involve the N-terminal (noncollagen) region of each chain, as the collagen structure itself can equally well accommodate three $\alpha 1$ strands. The cysteine contents of pro $\alpha 1$ and pro $\alpha 2$ (Table 1) are different. A part of this cysteine may be involved in determining the structure of the N-terminal region of individual precursor chains, for the following reason. Reduction with $\text{HSCH}_2\text{CH}_2\text{OH}$ alters the chromatographic behavior of both pro $\alpha 1$ and pro $\gamma 112$ (peaks I and II of Fig. 3), so that higher ionic strength buffers are required for elution. They also behave as more hydrophobic molecules than before reduction. In contrast, these treatments do not significantly alter the chromatographic behavior of partly degraded pro $\alpha 1$ (prepared by prolonged extraction of calvaria), which also has a lower cysteine content. We tentatively propose that the N-terminal regions of undegraded individual pro $\alpha 1$ chains are either held in one or more intrastrand loops by S-S linkage, or that some other material is attached to them by disulfide bonds. The same property is maintained when the pro $\alpha 1$ strands become components of pro $\gamma 112$.

Our preliminary finding that the proportion of trimerically disulfide-linked procollagen increases with the time the molecules spend within the cell suggests that the requisite juxtapositioning of SH groups is not assured at earlier times. We conclude that such groups can, however, be brought into register and linked by the (oxidative) action of oxidized glutathione plus diamide. These experiments were performed for 18 hr at 0° , and these conditions probably allowed thermal rearrangement of the relative positions of the registration peptides.

We propose that similar processes occur in the self assembly of procollagen molecules. An initial folding of the N-terminal region of each separate pro α chain may be followed by selec-

tive association into a triple-stranded structure that is neither covalently linked nor in proper register. Noncovalent forces between the N-terminal regions would play a primary part in this association. Only after relative diffusion of the components of this triplet would the correct alignment of the N-terminal regions be chanced upon, and when it is, then disulfide bonds would be formed to lock this arrangement. Subsequent detailed folding of the collagen triple helix would complete the assembly. In distinction to an undefined "stabilization" of the procollagen molecule by S-S links, proposed by others (2, 3, 5), we suggest that formation of these crosslinks serves two successive purposes: (i) selection of the correct alignment and (ii) strengthening of the association of the three chains against thermal disruption. The latter function is equivalent to "stabilization" but is probably not essential for assembly, as our oxidative experiments with glutathione clearly show that three procollagen chains can be closely associated in a nearly correct configuration without disulfide linkage. In summary, we propose primarily an ordering function for S-S formation rather than an enthalpic contribution to the stability of the pro $\gamma 112$. Disulfide-bridge formation might have a similar ordering role in other multimolecular self-associating systems.

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