COLLAGEN SYNTHESIS BY CELLS II: SECRETION OF A DISULFIDE LINKED MATERIAL

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SUMMARY: In medium from cultured chick embryo fibroblasts a species of collagen molecule was found which contained chains that were like α_1, but were larger and contained cysteine. Pepsin treatment or reduction of disulfide linkages was required for separation of the components of this species. The pepsin treatment gave rise to α sized collagen chains and this suggests a triple helical collagen structure for the original material.

Cultured fibroblasts secrete an appreciable portion of recently made native collagen into the culture medium where it remains in solution (1). In some of the molecules unknown internal cross links hold the constituent chains together and these molecules have been proposed as collagen precursors (2). We report here that these molecules contain disulfide linkages and on reduction yield pro α_1 type chains which contain cysteine. Precursor collagen molecules containing chains larger than alpha chains have been reported (3,4,5,6,7) and some contain cysteine (8,9). Our findings imply one function for the cysteine.

EXPERIMENTAL: Confluent layers of second passage chick embryo fibroblasts were cultured as described previously (1). Twenty-four hours before labelling fresh complete medium containing 100ug/ml ascorbic acid was added. Cells were incubated with radioactive amino acids (5-25uC/ml of 5^3H proline 34C/mM, or ^{14}C(U) proline 10mC/mM, or ^{3}H cystine 3.9 C/mM) for 24 hr. in fresh medium with ascorbic acid but without serum. The medium from the cultures was exhaustively dialyzed at 4° against 0.5M CH_3COOH and then against 0.16M CH_3COONa, adjusted to pH 4.8 with CH_3COOH.

Carboxymethylcellulose (CMC) chromatography was carried out by the method of Piez et al. (10) modified by inclusion of 6M urea in all buffers.

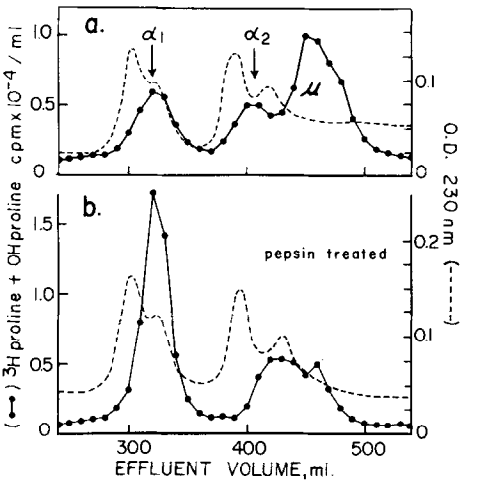

Figure 1. CMC chromatograms of equal aliquots of culture medium labelled with ^3H proline for 24 hr: (a) before and (b) after digestion with pepsin. Rat tail tendon collagen, 7 mg, was added to each sample and gave the optical density patterns shown. Control experiments with isolated chick α_1 and α_2 showed that these eluted in the indicated positions relative to the rat collagen chains. Radioactivity of 0.5ml aliquots of each 10ml fraction was measured by scintillation counting (cpm/dpm = 0.4). The ordinate values in (a) are ((cpm/ml, measured) x (0.72/0.58)) and the ordinate values of (b) are without correction. In experiment (a) 58% of the initial radioactivity was recovered in the total chromatogram, and 72% in experiment (b). (•) cpm/ml; (----) O.D. 230nm.

To the sample three volumes of deionized 8M urea solution were added. The pH value was adjusted to 4.8 and the mixture was heated to 40° for 30 min. Chromatograms (Fig.1a) show peaks corresponding to α_1 and α_2 collagen chains and a prominent peak following the α_2 peak. This peak, designated μ, is not completely resolved from the α_2 peak. This material elutes in the same position on rechromatography. Collagen is the prime constituent of μ since it has a high hydroxyproline content ((Hypro/ (Hypro + Pro) = 0.40)). It is consistently made both by confluent and non-confluent cultures under a variety of conditions.

Digestion of the culture medium, dialyzed into 0.5M CH_3COOH, with 100 ug/ml pepsin at 15° for 6 hr. abolishes peak μ (Fig.1b) and causes transfer of its radioactive material to the α_1 and α_2 regions of the chromatograms, approximately in proportion 2:1 (Table 1). Acrylamide gel

TABLE 1

	μ	α_1 region	α_2 region	total
(a) untreated	4.36	2.3	1.75	8.4
$\left\{\begin{matrix}0.67\mu \rightarrow \alpha_1 \\ 0.33\mu \rightarrow \alpha_2\end{matrix}\right\}$	↳	2.9	1.45	
expected	-	5.2	3.2	
(b) pepsin treated	-	5.3	3.6	8.9

Radioactivity (cpm x 10^{-5}) found in the different regions of CMC chromatograms shown in Fig. 1a and 1b is given in rows (a) and (b). The effect of redistributing the radioactive material of peak μ between regions α_1 and α_2 in an expected proportion of 2:1 is shown.

electrophoresis (10% acrylamide, 0.5% sodium dodecyl sulfate, 6M urea, pH 8.8 modified from (11))shows these products to migrate with α chains.

Peak μ is also abolished if the dialyzed culture medium is treated with β-mercaptoethanol ($HSCH_2CH_2OH$) (12), or with 190ug/ml $NaBH_4$ in phosphate buffer pH 7.5, at 22° for 18 hr. Both reactions were followed by carboxymethylation with excess ICH_2COOH. Controls showed that carboxymethylation alone, without reduction, has no effect on the chromatographic pattern and that the material was unaltered after being kept at pH 8.6 for 24 hr. at 22°.

Reduction of chromatographically isolated μ with $HSCH_2CH_2OH$ or $NaBH_4$ followed by carboxymethylation and CMC chromatography shows that the μ peak is replaced by two peaks: one is coincident with α_1 and the other is in the α_2 region (Fig.2). We denote these materials as pseudo alpha 1 ($\psi\alpha_1$) and pseudo alpha 2 ($\psi\alpha_2$) to indicate their chromatographic similarity to α_1 and α_2. Acrylamide gel electrophoresis of $\psi\alpha_1$ together with markers labelled with another isotope shows that it behaves as a larger polypeptide than α_1 and migrates like pro α_1 collagen chains prepared from chick calvaria (3),(Fig.3).

CMC chromatography of media from cultures labelled with ^{14}C proline and ^{3}H cystine shows incorporation of cystine into μ, but not into α_1 and α_2 peaks. The ^{3}H cystine labelled material from the μ region was passed

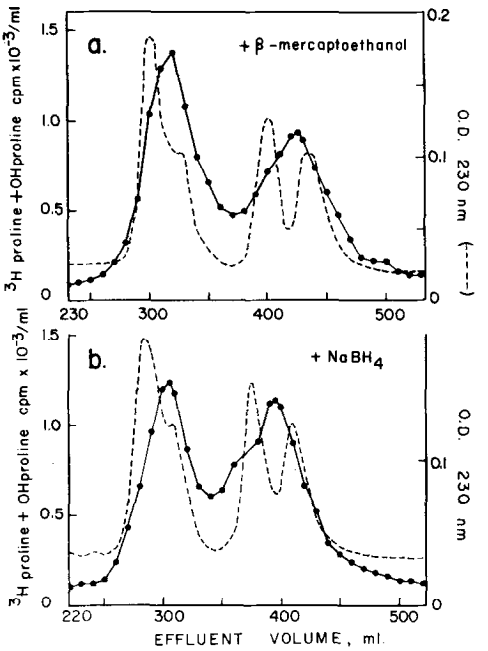

Fig. 2.

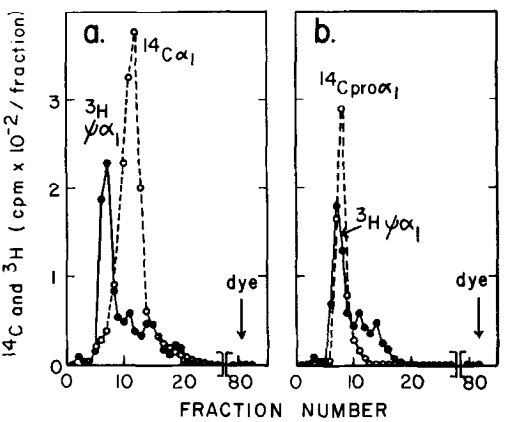

Fig. 3.

Figure 2. Material from the μ region of a chromatogram such as Fig. 1a was pooled and divided into equal parts. One was reduced with $HSCH_2CH_2OH$ (a), the other with $NaBH_4$ (b), and after carboxymethylation and addition of rat tail tendon collagen both were chromatographed as before on CMC. (•) cpm/ml; (----) O.D. 230nm.

Figure 3. Acrylamide gel electrophoretograms of $\psi\alpha_1$ (^3H) (•), together with ^{14}C marker collagen chains (o); (a) α_1 (^{14}C) isolated from culture medium as in Fig. 1a; (b) pro α_1 (^{14}C) isolated from chick calvaria. Gels were 12 cm long and were divided into 87 equal fractions. Radioactivity measurements were corrected for overlap between ^3H and ^{14}C disintegrations. Radioactivity was only found in the first 23 fractions. Arrows indicate positions of bromophenol blue marker.

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through a molecular sieve column of 6% agarose (Biogel A-5m, 6M urea, 0.04M acetate, pH 4.5). A test sample of β cbllagen chains eluted just in the void volume of this column. The ^3H cystine labelled material in the void volume was reduced with $HSCH_2CH_2OH$, carboxymethylated and rechromatographed on CMC with carrier collagen. A small but definite peak of ^3H material appeared in the α_1 region, but there was insufficient radioactivity to determine whether or not the α_2 region also contained cysteine. Further ^3H cysteine

containing material was eluted from the CMC column upon increasing the NaCl concentration of the eluting buffer to 1M.

DISCUSSION: We conclude that: (a) $\psi\alpha_1$ chains contain cysteine, (b) that probably there is other cysteine containing material in μ, and (c) that one or more disulfide linkages are essential to the stability of μ. The major portion of μ is resistant to pepsin before denaturation and this may indicate a native, triple helical collagen structure composed of two $\psi\alpha_1$ chains and one $\psi\alpha_2$ chain. The $\psi\alpha_1$ chain of μ has the same molecular size as a known collagen precursor which can be extracted from calvaria: pro α_1 (3,8), and both chains contain cysteine. $\psi\alpha_1$ differs from pro α_1, however, in that it co-chromatographs on CMC with α_1. Prior to reduction of disulfide linkages $\psi\alpha_1$ is totally absent from the α_1 region of CMC chromatograms. A relation of μ to early forms of collagen is also suggested by its presence in extracts of cells, and in culture medium after only 40 min. incubation with radioactive label. Fibroblasts begin to secrete radioactive collagen at this time (1).

Collagen may be associated with other materials within the cell. Enveloped packages of collagen (13,14) and microtubular transport of collagen (15,16) have been suggested. A complex of three collagen chains with such another material could be μ. An extra material carrying more positive charge than $\psi\alpha_1$ and $\psi\alpha_2$ is required if the behavior of μ on ion exchange chromatography is to be an average of its components. (Controls showed that reduction and carboxymethylation has very little effect, if any, on the CMC chromatographic properties of pro α_1 obtained from calvaria and of α_1 and α_2 from cell cultures). Such an extra component of μ might be found in the ^{3}H cysteine containing material which was isolated with μ, but which behaved, after cleavage of μ, as more positively charged material than $\psi\alpha_1$, $\psi\alpha_2$ or μ. Such material could, of course, also be held to collagen in other ways than by disulfide linkage. Linkages based on aldehydes, such as are found in other forms of collagen, are unlikely

both because $NaBH_4$ splits μ and because its formation is unaffected by addition of the lathyrogen $NH_2CH_2CH_2CN$ to cell cultures.

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