CONFORMATION OF UNDERHYDROXYLATED COLLAGEN SYNTHESIZED BY 3T6 FIBROBLASTS IN CULTURE

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1. Introduction

Although hydroxyproline occurs in all vertebrate interstitial collagens [1], it has been thought that it was not essential for the structural integrity of the collagen molecule [2,3] and its function has remained obscure. Previous experiments with embryonic tibiae [4] and fibroblasts [5–8] indicated that when the hydroxylation of proline and lysine was inhibited with α,α'-dipyridyl, unhydroxylated molecules were secreted at a markedly reduced rate. When tibiae [9–11] or fibroblasts [12,13] were incubated with analogues of proline, collagen molecules containing significantly decreased amounts of *trans*-hydroxyproline were synthesized. These molecules also seemed to be secreted much more slowly than normally and these experiments suggested that *trans*-hydroxyproline was a necessary requirement for normal secretion. Peterkofsky [14] has reported that ascorbate deficiency in fibroblasts in culture causes a significant decrease in the extent of proline hydroxylation and also decreases the rate at which collagen is secreted during a 4 hr-labeling period. Bates et al. [15] found that the underhydroxylated collagen synthesized by ascorbate deficient fibroblasts was more soluble relative to collagen synthesized by ascorbate supplemented cultures during a 24 hr-labeling period. Recent experiments using short pulse [16] labeling of fibroblasts and pepsin digestion at 15°C as an enzymatic probe of collagen conformation have demonstrated that triple helix formation occurs intracellularly before collagen is secreted. Pepsin digestion studies at higher temperatures have revealed that purified unhydroxylated collagen thermally denatures between 20°C and 25°C while hydroxylated collagen is stable at least to 35°C [17]. This latter finding suggested that hydroxyproline in collagen contributes significantly to the thermal stability of the triple helix and that hydroxyproline deficient molecules may be denatured in cells and tissues at 37°C. The present study demonstrates that hydroxyproline deficient collagen molecules produced by cultured fibroblasts at 37°C under three different experimental conditions are found as denatured chains in the cultures.

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2. Materials and methods

The mouse fibroblast line 3T6 was grown as previously described [5]. Further details of incorporation experiments and analytical procedures are given in the table and figures. Crystalline pepsin was purchased from Sigma Chemical Co. Bacterial collagenase from Worthington Biochemical Corp. was further purified by DEAE-chromatography and then isoelectric focusing [18]. It contained no non-specific proteolytic activity when tested with a number of different non-collagenous proteins. Radiochemicals and other materials were obtained as previously described [12].

3. Results

Under the control growth conditions employed here, more than 75% of the collagen synthesized by these late log phase cells during an 8 hr-labeling period was found in the medium (table 1). When the cells were incubated with 1 mM α,α'-dipyridyl or in an atmosphere of 90% N_2 and 10% CO_2, the hydroxylation of proline was inhibited by greater than 90%. In addition, there was a significant inhibition of total incorporation in both the cell layer as well as the medium fractions. When the cells were grown in the absence of ascorbate, the fraction of the counts in the medium which were in [^{14}C]hydroxyproline (degree of hydroxylation) decreased to 4.4% compared to the ascorbate supplemented control value of 32.5%. There was a moderate decrease in the quantity of total ^{14}C in the medium of ascorbate deficient cultures, but as contrasted with treatment with α,α'-dipyridyl and oxygen deprivation, there was a negligible decrease in the total ^{14}C incorporation in the cell layer (table 1).

The continued finding of significant amounts of labeled protein in the media under all three experimental conditions lead us to examine further the nature of the secreted proteins. Under control conditions, approx. 52% of the incorporated [^{14}C]proline in the medium was found in proteins of mol. wt. 95,000–120,000 when examined by sodium dodecyl sulphate (SDS)–polyacrylamide electrophoresis (fig. 1A). We have found variation in the relative amounts of procollagen and α chains in the medium of cells grown under apparently identical conditions (compare undigested control samples in figs. 1A and 2A). When the control medium proteins were digested with highly purified collagenase before electrophoresis, essentially no radioactivity was recovered in fractions corresponding to 95,000–120,000 daltons. The distribution of radioactivity in the labeled media proteins of cells incubated under the three experimental condi-

Table 1
Incorporation of [^{14}C]proline into proteins in the cell layer and medium and [^{14}C]hydroxyproline content of these proteins.

Incubation conditions during labeling of cells	Cells		Medium	
	Total ^{14}C (DPM × 10^{-3})	Degree of hydroxylation* (%)	Total ^{14}C (DPM × 10^{-3})	Degree of hydroxylation* (%)
Control	552.4	1.3	91.0	32.5
Ascorbate deficient	537.2	0.8	56.5	4.4
10^{-3} α,α'-dipyridyl	230.8	<0.3	31.9	< 0.3
Anaerobic	228.5	1.3	23.1	1.5

* Values are 100 × [^{14}C]hydroxyproline per total ^{14}C.
7×10^5 cells were plated onto 50 mm petri dishes in medium supplemented with 10 μg/ml ascorbic acid and 48 hr later 25 μg/ml ascorbate was added to the plates. This procedure was necessary since we found that cells did not plate efficiently at ascorbate concentrations greater than 10 μg/ml. Twelve hours later experiments were started. The medium was replaced with fresh medium containing 1 μCi[^{14}C]proline and the incubation continued for 8 hr. At the end of the incubation, medium and cells were dialyzed separately and the proteins analyzed for total ^{14}C incorporation and [^{14}C]hydroxyproline content [19]. The values represent the average of duplicate platings. Special conditions: (i) Ascorbate deficient cells were never supplemented with ascorbate; (ii) 1 mM α,α'-dipyridyl was added 20 min before the isotope addition; (iii) the 90% N_2–10% CO_2 incubation began 1 hr before isotope addition.

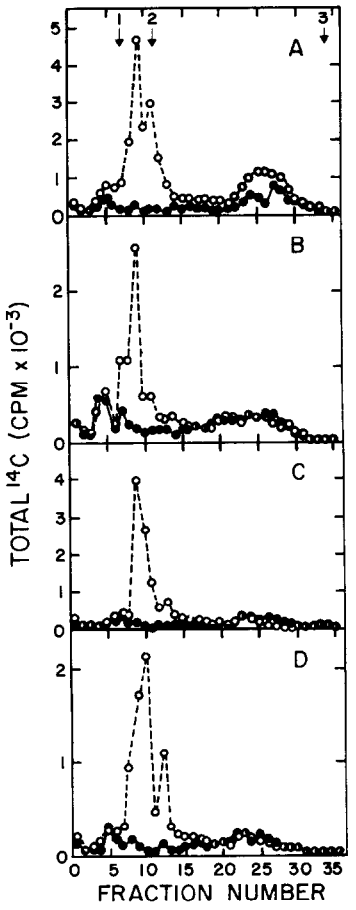

Fig. 1. Gel electrophoresis of labeled media proteins secreted by: A) control cells; B) ascorbate deficient cells; C) cells incubated with 1 mM α,α'-dipyridyl; D) anaerobic cells; all after incubation with and without collagenase. Cells were labeled with [^{14}C]proline as described in table 1. At the end of the incubation, the media were removed, 0.05 vol 1 M Tris, pH 7.4, was added and aliquots incubated with or without 4 μg/ml purified collagenase at 37°C for 5 hr. At the end of the digestion, SDS and mercaptoethanol were added to final concentrations of 1% and the samples were immediately placed in a boiling water bath for 2 min. The samples were then dialyzed against 0.01 M $NaPO_4$, pH 7.0, 0.1% SDS, and 0.1% mercaptoethanol and aliquots then run on 5% polyacrylamide gels containing half the standard amount of cross-linker. The gels were fractionated and counted [12]. Incubation with collagenase (•-•-•); without collagenase (o-o-o). 1) rat tail β chains; 2) rat tail α chains; 3) tracking dye.

tions designed to limit hydroxylation was similar to that of the control. The labeled protein in the 95,000–120,000 dalton region was also sensitive to bacterial collagenase (fig. 1). The labeled collagen in the media was isolated by gel filtration on Biogel A-5 Agarose in SDS [7]. The collagen had a degree of hydroxylation of 43.7% for the ascorbate supplemented control, 4.7% for the ascorbate deficient culture, and less than 1% for the α,α'-dipyridyl treated or anoxic cultures (chromatograms not shown). From these experiments we conclude that significant amounts of labeled collagen of normal molecular weight can be secreted by these cells even when the hydroxylation of proline is essentially completely inhibited, although the rate of secretion is probably decreased [5,8,14].

In order to compare the conformations of the collagen secreted by the control and treated cultures, we digested the secreted proteins in the media with pepsin at 15°C immediately (to prevent artifactual triple helix formation) after removing the media from the cells. It has been well established that pepsin at 15°C does not digest the triple helical portion of the collagen molecule but only the non-helical moiety [20,21]. Denaturated chains are completely degraded. After incubation with pepsin, most of the label in the control proteins was found in a single peak which co-electrophoresed with marker α chains (fig. 2). Thus, this collagen in the control medium was triple-helical. In contrast, the underhydroxylated collagen found secreted into the medium under the three experimental conditions was entirely digested by pepsin at 15°C and large amounts of radioactivity appeared in low molecular weight peptides which electrophoresed near the tracking dye and which represent digestion products.

4. Discussion

Since Jimenez et al. [17] found that unhydroxylated collagen thermally denatured between 20 and 25°C, they suggested that such unhydroxylated collagen may exist predominantly as denatured chains at normal body temperatures. The present work supports this idea. Although significant amounts of normal molecular weight collagen were found in the media of cells in which the hydroxylation of proline was severely limited by removing O_2, Fe^{2+} or ascorbate, three different known cofactors of proline hydroxylase, the chains were in a conformation which

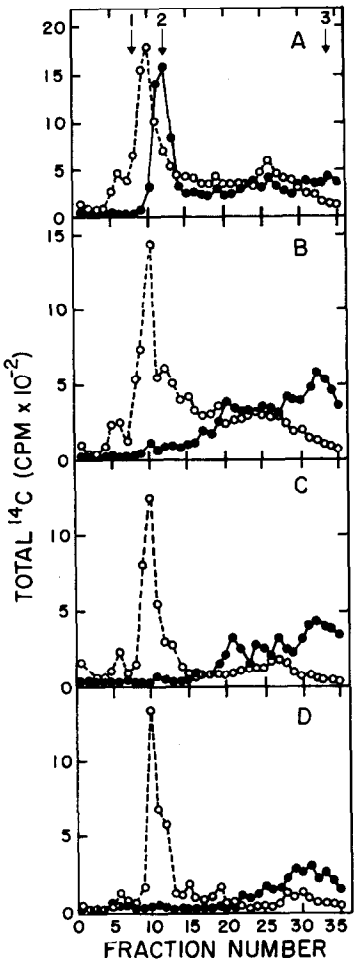

Fig. 2. Gel electrophoresis of labeled media proteins secreted by: A) control cells; B) ascorbate deficient cells; C) cells incubated with 1 mM α,α'-dipyridyl; D) anaerobic cells; all after incubation with and without pepsin. Cells were labeled with [^{14}C]proline as described in table 1. At the end of the incubation, the media were removed and titrated immediately at room temperature to pH 2.0 with 1 M HCl. Aliquots were then incubated with and without 100 μg/ml for 6 hr at 15°C. At the end of the digestion the solutions were neutralized and electrophoresed as in fig. 1. Incubation with pepsin (●-●-●); without pepsin (○-○-○). 1) rat tail β chains; 2) rat tail α chains; 3) tracking dye.

rendered them susceptible to pepsin at 15°C and they were therefore probably not triple-helical. The collagen in the media probably did not arise from nonspecific leakage of labeled proteins from the cells, since the distribution of radioactivlty in the cell layer proteins was much more heterogeneous (electrophoretic patterns not shown). Contamination of the secreted proteins would have been detected by alteration of the electrophoretic pattern of the media proteins. Ramaley and Rosenbloom [5] have previously reported the absence of normal size collagen molecules in the media of cultures in which proline hydroxylation was maximally inhibited with α,α'-dipyridyl. It is likely that the denatured chains now known to be present in such media were selectively lost or degraded in the handling prior to the Agarose chromatography which was performed in 1 M $CaCl_2$.

Since triple-helix formation normally occurs intracellularly before collagen is secreted [16] it is possible that prevention of such helix formation by reduction in the hydroxyproline content could cause a significant decrease in the rate of secretion. This notion is consistent with the observation that when some amino acid analogues of proline are incorporated into collagen they disrupt triple-helix formation and cause intracellular retention of the abnormal collagens [12,13]. The present results may also explain the well known disruption of normal collagen fibril formation in scorbutic animals as well as the increased solubility of collagen noted by Bates et al. [15] in ascorbate deficient cultures of 3T6 fibroblasts. The more slowly secreted, denatured chains would be more soluble, could not form fibrils, and would be more susceptible to nonspecific digestion.

Acknowledgements

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References

[1] J.E. Eastoe, Treatise on Collagen, Vol. 1, Ed. G.N. Ramachandran (Academic Press, New York, London, 1967) p. 1.
[2] R.E. Burge and R.D. Hynes, J. Mol. Biol. 1 (1959) 155.
[3] K.A. Piez and J. Gross, J. Biol. Chem. 235 (1960) 995.
[4] K. Juva, D. Prockop, G. Cooper and J. Lash, Science 152 (1966) 92.
[5] P.B. Ramaley and J. Rosenbloom, FEBS Letters 15 (1971) 59.

[6] P. Dehm and D.J. Prockop, Biochim. Biophys. Acta 240 (1971) 358.
[7] S. Jimenez, P. Dehm. B.R. Olsen and D.J. Prockop, J. Biol. Chem. 248 (1973) 720.
[8] R.L. Margolis and L.N. Lukens, Arch. Biochem. Biophys. 147 (1971) 612.
[9] T. Takeuchi and D.J. Prockop, Biochim. Biophys. Acta 175 (1969) 142.
[10] J. Rosenbloom and D.J. Prockop, J. Biol; Chem. 245 (1970) 3361.
[11] J. Rosenbloom and D.J. Prockop, J. Biol. Chem. 246 (1971) 1549.
[12] M. Harsch, L. Murphy and J. Rosenbloom, FEBS Letters 26 (1972) 48.
[13] J. Uitto, P. Dehm and D.J. Prockop, Biochim. Biophys. Acta 278 (1972) 601.
[14] B. Peterkofsky, Biochem. Biophys. Res. Commun. 49 (1972) 1343.
[15] C.J. Bates, A.J. Bailey, C.J. Prynne and C.I. Levene, Biochim. Biophys. Acta 278 (1972) 372.
[16] J. Rosenbloom, M. Harsch and D. Hardman, Federation Proc. 32 (1973) 649.
[17] S.A. Jimenez, M. Harsch and J. Rosenbloom, Biochem. Biophys. Res. Commun., in press.
[18] M. Miyoshi and J. Rosenbloom, submitted for publication.
[19] K. Juva and D.J. Prockop, Anal. Biochem. 15 (1966) 77.
[20] A.L. Rubin, D. Pfahl, P.T. Speakman, P.J. Davison and F.O. Schmitt, Science 139 (1963) 37.
[21] D.L. Layman, E.B. McGoodwin and G.R. Martin, Proc. Natl. Acad. Sci. U.S. 68 (1971) 454.