HYDROXYPROLINE STABILIZES THE TRIPLE HELIX OF CHICK TENDON COLLAGEN*

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SUMMARY:

The thermal stability of unhydroxylated procollagen relative to hydroxylated procollagen was investigated using pepsin digestion at various temperatures in the interval 15° to 35° as an enzymatic probe of conformation. The results demonstrate that the unhydroxylated molecules thermally denature between 20° and 25°, while the hydroxylated molecules are stable at least to 35°. This finding suggests that the presence of hydroxyproline in the molecule contributes significantly to the thermal stability of collagen. The results also suggest that triple strand formation may be required for normal secretion.

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 remains obscure. Previous experiments with embryonic tibiae (4) and fibroblasts (5, 6, 7) indicated that when the hydroxylation of proline and lysine was inhibited, unhydroxylated molecules were secreted at a markedly reduced rate. In the case of the fibroblasts, the retained molecules appeared to be slowly degraded intracellularly. When tibiae (8-10) or fibroblasts (11, 12) 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

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that trans-hydroxyproline was a necessary requirement for normal secretion. Recent experiments using short pulse (13) or longer (7) labeling of fibroblasts and pepsin digestion at 15° as an enzymatic probe of collagen conformation have demonstrated that triple helix formation occurs intracellularly before collagen is secreted. Studies of unhydroxylated collagen (7) have shown that this hydroxyproline deficient collagen forms a triple helical structure which is indistinguishable from normal hydroxylated collagen by the criteria of pepsin digestion at 15° and segment long spacing formation at 4°. The present study reveals, however, that the unhydroxylated collagen is much less thermally stable than its hydroxylated counterpart and this suggests that the presence of hydroxyproline in the molecule contributes significantly to collagen thermal stability.

MATERIALS AND METHODS

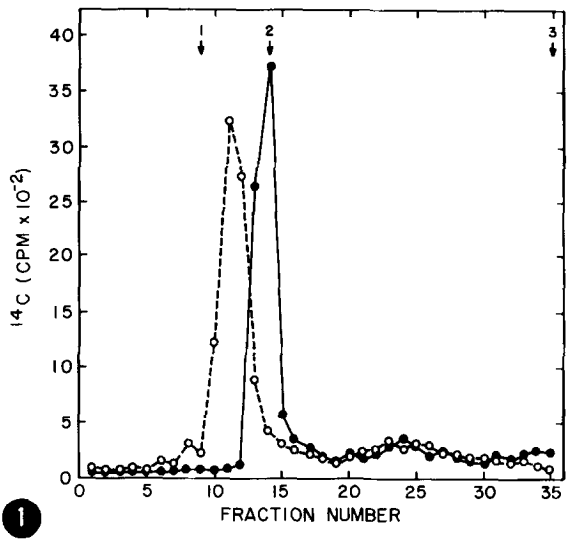
Fibroblasts were isolated from 17 day old chick embryo tendons as described by Dehm and Prockop (6) except that the digestion with bacterial collagenase and trypsin was performed in Eagles minimum essential medium containing 2% fetal calf serum. Subsequently 10^7 cells/ml were incubated in Krebs medium containing 2% serum, 25 µg/ml ascorbic acid, ^{14}C-proline, and either 10^{-6} M colcemide or 5 X 10^{-4} M α,α'dipyridyl as required. After the incubation of the cells at 37°, the suspension was rapidly chilled and centrifuged for 2 minutes at 1500 x g. The cells were resuspended in the same ice cold incubation medium minus the isotope plus 100 µg/ml cycloheximide to stop protein synthesis and the cells were recentrifuged. The cell pellet was then resuspended in a volume of 0.5 M acetic acid equal to that of the original incubation volume and the suspension was shaken for 18 hours at 4° to extract the collagen. The suspension was then centrifuged at 12,000 x g for 15 minutes. Aliquots of the clear supernatant were then used directly for incubation with or without a final concentration of 100 µg/ml of pepsin which had pre-

viously been dissolved in 0.5 M acetic acid. At the end of the digestions which were carried out at 15, 20, 25, 30, and 35 degrees for 6 hours, the solutions were neutralized, sodium dodecyl sulfate[i] and mercaptoethanol were added to final concentrations of 1% and the samples were immediately placed in a boiling water bath for 2 minutes to completely inactivate the pepsin and allow the collagen to rapidly bind the SDS. The samples were then prepared for SDS disc gel electrophoresis and run as previously described (11). The gels were immediately chilled and cut into 1.5 mm fractions in order to minimize losses and the fractions were solubilized with 30% H_2O_2 and counted in a scintillation counter (11). Duplicate gels were run containing radioactive samples and purified α and β rat tail collagen. These gels were stained with Coomasie blue and the radioactive peaks located relative to these markers. The position of the radioactive peaks was identical in the stained and unstained gels when corrected for gel swelling during destaining.

RESULTS AND DISCUSSION

When the unhydroxylated collagen which had been incubated without pepsin at 20° to 35° was electrophoresed on SDS polyacrylamide gels, about 63% of the total radioactivity was found in a single sharp peak having a molecular weight of approximately 125,000, which is consistent with a form of unhydroxylated procollagen. After pepsin digestion at 20°, most of the radioactivity was now found in a peak which coincided with the position of single α chains of 95,000 daltons. Approximately 90% of the counts originally found in the 125,000 dalton peak were recovered in the 95,000 dalton peak (Table I and Fig. 1). These results are consistent with the previous findings of Jimenez et al. (7) who performed the pepsin digestion at 15°. In contrast, when the incuba-

[i] Sodium dodecyl sulfate is abbreviated SDS



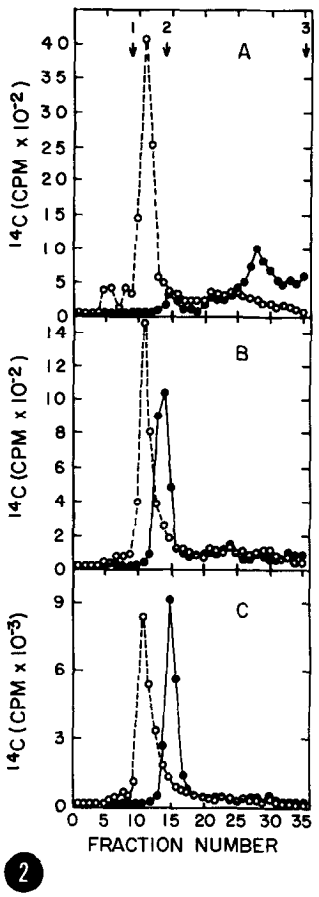


Figure 1. Gel electrophoresis of unhydroxylated procollagen after incubation with and without pepsin at 20° for 6 hours. Aliquots of unhydroxylated procollagen prepared from cells incubated with α, α'-dipyridyl for two hours were incubated with and without 100 µg/ml pepsin in 0.5 M acetic acid for 6 hours. At the end of the digestion the solutions were neutralized, SDS and mercaptoethanol were added to final concentrations of 1% and the samples immediately placed in a boiling water bath for 2 minutes. 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 (11). The gels were fractionated and counted as described in Materials and Methods. Incubation with pepsin (●-●-●); without pepsin (o-o-o). 1, rat tail β chains; 2, rat tail α chains; 3, tracking dye.

Figure 2. Gel electrophoresis of unhydroxylated collagen (A), collagen from 30 minute pulsed cells (B), and collagen from colcemide treated cells (C) after incubation with and without pepsin at 25° for 6 hours. Incubation conditions and gel electrophoresis as in Fig. 1. With pepsin (●-●-●); without pepsin (o-o-o). 1, rat tail β chains; 2, rat tail α chains; 3, tracking dye.

tions were carried out at 25°, a small peak containing less than 10% of the original counts was recovered in this 95,000 dalton region (Fig. 2), and at higher temperatures less than 5% of the counts were so recovered

Table I

RECOVERY OF RADIOACTIVITY IN PROCOLLAGEN AND α CHAINS AFTER INCUBATION OF COLLAGEN PREPARATIONS WITH AND WITHOUT PEPSIN

The values listed in the table were derived by summing the observed counts in the peaks illustrated in Fig. 1, 2 and 3 as well as from similar data at 30° which is not shown.

Source of Substrate	Temp °C	^{14}C-procollagen[a] cpm X 10^{-2}	^{14}C-α chain[b] cpm X 10^{-2}	$\frac{^{14}\text{C-}\alpha \text{ chain}}{^{14}\text{C-procollagen}} \times 100$ %
α, α' dipyridyl treated	20	82.7	74.8	90.4
	25	88.8	8.5	9.6
	30	88.7	3.8	4.3
	35	94.7	3.5	3.7
30 min. pulse	20	34.8	25.5	73.2
	25	33.4	26.0	77.7
	30	36.8	27.3	74.2
	35	37.5	26.6	71.1
Colcemide treated	20	193.6	184.1	95.1
	25	200.1	194.8	97.3
	30	187.3	173.5	92.6
	35	192.4	203.7	105.9

[a]Five peak fractions in Fig. 1, 2, and 3 incubations without pepsin.

[b]Five peak fractions in Fig. 1, 2, and 3 incubations with pepsin.

(Table I and Fig. 3). However, a large amount of radioactivity appeared in low molecular weight peptides which electrophoresed near the tracking dye and which represent digestion products.

Two types of control samples were studied in order to demonstrate that hydroxylated collagen recovered from inside the cells was not unusually susceptible to pepsin. The first type was obtained from cells which had been simply incubated for 30 minutes with ^{14}C-proline. Previous results have shown that the intracellular procollagen after this

period of incubation is fully hydroxylated and that 70% to 80% of the total radioactivity electrophoresing in the procollagen region was converted to α chain size by pepsin digestion at 15° (13). In order to compare the unhydroxylated collagen to a form of hydroxylated collagen which had been retained intracellularly for approximately the same time, cells were incubated with 10^{-6} M colcemide and ^{14}C-proline for two hours. This collagen synthesized in the presence of colcemide has been shown to be fully hydroxylated but is secreted at a markedly reduced rate (14). The results with both these control procollagen samples were qualitatively similar (Table I and Fig. 2 and 3). Between 71% and 78% of the radioactivity in the procollagen peak from the 30 minute pulsed cells was recovered in the α chain region after pepsin digestion at all temperatures in the interval 20° to 35°. There was essentially quantitative transfer from the procollagen to α chains when the substrate synthesized in the presence of colcemide was incubated with pepsin under the same conditions. We attribute this difference in the controls to the intracellular accumulation of triple helical procollagen in the case of the colcemide treated cells relative to the 30 minute pulsed cells.

Although it was originally suggested by Gustavson (15) on the basis of thermal shrinkage of a number of skin collagens that hydroxyproline through hydrogen bonding could stabilize the collagen structure, later comparative studies by Burge and Hynes (2) and Piez and Gross (3) suggested that the total imino acid content was the dominant stabilizing factor. There are no previously published experiments comparing the stability of hydroxylated collagen relative to its unhydroxylated equivalent. Earlier studies with pepsin digestion of native collagen demonstrated that only the non-helical segments or "telopeptides" were cleaved and that the triple-helical portion was unaffected (16). This result has been subsequently amply substantiated with different types of radioactively

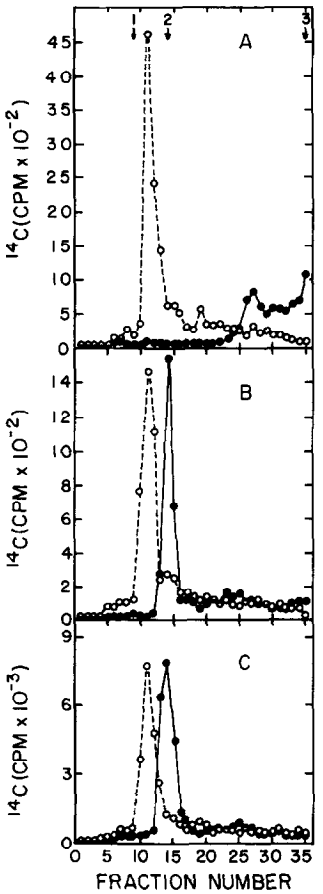


Figure 3. Gel electrophoresis of unhydroxylated collagen (A), collagen from 30 minute pulsed cells (B), and collagen from colcemide treated cells (C) after incubation with and without pepsin at 37° for 6 hours. Incubation conditions and gel electrophoresis as in Fig. 1. With pepsin (●-●-●); without pepsin (o-o-o). 1, rat tail β chains; 2, rat tail α chains; 3, tracking dye.

labeled procollagen samples (17, 18). After denaturation, the entire collagen molecule is degraded by pepsin. From our experiments, we can conclude that the unhydroxylated collagen at temperatures of 25° or higher is in a conformation which renders it susceptible to pepsin while hydroxylated molecules retain their resistance at least to 35°, the latter result being consistent with published optical rotation studies on chick collagen (19). At present, the most reasonable explanation of our data is that proline hydroxylation confers added thermal stability to the triple stranded helix and thus renders it more resistant to pepsin di-

gestion. Experiments are now in progress to obtain sufficient amounts of unhydroxylated collagen for optical studies of its thermal denaturation properties. Our results tend to favor structure I of Rich and Crick (20) since this structure can accommodate a hydrogen bond by hydroxyproline in position 3 to a neighboring chain within the 3 chain group while structure II cannot.

A number of studies have suggested that when the hydroxylation of collagen is inhibited by α,α' dipyridyl, the unhydroxylated molecules accumulate intracellularly and are not secreted at a normal rate (4-7). Our results suggest that the unhydroxylated molecules may be single stranded intracellularly at 37° and that they may not be secreted at the same rate as hydroxylated triple helical molecules. This hypothesis is consistent with the finding 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 (11, 12). The results reported here with colcemide indicate, however, that this drug acts by some other mechanism than disruption of triple strand formation since the collagen synthesized in its presence was stable to pepsin at 35°. The retention mechanism may be through disruption of microtubules as originally suggested (14).

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