SYNTHESIS AND SECRETION OF UNDER-HYDROXYLATED PROCOLLAGEN AT VARIOUS TEMPERATURES BY CELLS SUBJECT TO TEMPORARY ANOXIA

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Summary: Cells isolated by enzymic digestion of embryonic tendon were incubated under N_2 so that they synthesized and accumulated the unhydroxylated form of procollagen which is known as protocollagen and which is largely comprised of pro-α chains linked by interchain disulfide bonds. The cells were then exposed to O_2 so that the intracellular protocollagen was hydroxylated and secreted as procollagen. When the hydroxylation was allowed to proceed at 31° or 34°, the procollagen secreted into the medium was triple-helical but its hydroxyproline content was less than two-thirds and its hydroxylysine content was less than half the control. Even when the hydroxylation was allowed to occur at 37°, the procollagen secreted by the cells was under-hydroxylated by about 15% in terms of its hydroxyproline content and about 45% in terms of its hydroxylysine content. The results may have consequences for collagen synthesis by tendons and similar tissues in vivo, since temporary anoxia in such tissues may well lead to the synthesis of a less stable procollagen or to fibers of decreased tensile strength.

Several factors appear to determine the content of hydroxyproline and hydroxylysine of collagen, since there are no messenger-RNA codons for either of these amino acids and they are synthesized by post-translational hydroxylation of prolyl and lysyl residues in the "Y-position" of the repeating -X-Y-Gly- sequences of the polypeptide chains (for recent reviews, see 1, 2).

Abbreviation: SDS, sodium dodecyl sulfate.

When the prolyl and lysyl hydroxylases in freshly-isolated connective tissues or in cells from such tissues are inhibited with α,α′-dipyridyl or with anaerobic conditions, the cells synthesize and accumulate protocollagen, the unhydroxylated form of procollagen (1,3-10). If inhibition of the hydroxylases is reversed in 1 or 2 hr by addition of Fe^{2+} or by exposing the cells to O_2, most of the accumulated protocollagen is hydroxylated and secreted (1,3,4,10). In the present paper, we examined the question of whether subjecting cells isolated from embryonic tendon to temporary anoxia can influence the degree to which the procollagen molecule is hydroxylated before being secreted.

Materials and Methods. Cells were prepared by enzymic digestion of leg tendon of 17-day old chick embryos and were incubated in modified Krebs medium (11). Unless otherwise indicated, experimental conditions and sources of materials were the same as previously specified (10-12).

Degree of Hydroxylation of Proline and Lysine After Intracellular Hydroxylation of Protocollagen. To accumulate intracellular protocollagen, cells isolated from chick embryo tendons by controlled enzymic digestion (11) were pulse-labeled with [^{14}C]proline under N_2 for 90 min at 37° (10). Further protein synthesis was then inhibited by adding cycloheximide, and 10 min later the inhibition of the hydroxylases was reversed by exposing the system to atmospheric O_2 for 120 min. The [^{14}C]pro-α chains of procollagen secreted into the medium were then isolated by gel filtration and polyacrylamide gel electrophoresis in SDS (10,12). If the incubation under O_2 was performed at 37° (Fig. 1A), the

ratio of [^{14}C]hydroxyproline to [^{14}C]hydroxyproline plus [^{14}C]proline in isolated pro-α chains was 33 to 37% in six experiments. In the same experiments, the value for this ratio was 41 to 43% when the cells were incubated at 37° under air throughout the labeling period (Fig. 1A).

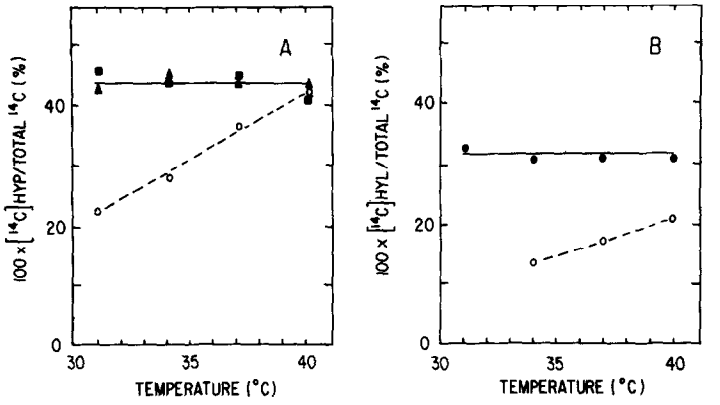

Figure 1. To produce temporary anoxia, 1.4 x 10^9 tendon cells (11) in 40 ml of modified Krebs medium containing 10% fetal calf serum were incubated as described previously (10) under N_2 at 37° with 20 μCi of [^{14}C]proline or 30 μCi of [^{14}C]lysine for 90 min. Cycloheximide, 4.2 mg in 1 ml of medium containing 10% fetal calf serum, was then added and the flasks were equilibrated for 10 min to the temperatures indicated. The cells were exposed to O_2 for 120 min, 4.2 ml of medium containing 11 mM α,α´-dipyridyl was added, and the medium was separated by centrifugation. The medium was dialyzed at 4° against 0.4 M NaCl and 0.1 M Tris, pH 7.5, and the ^{14}C-protein was precipitated with 176 mg of ammonium sulfate per ml. The ^{14}C-protein was treated with SDS-mercaptoethanol and the pro-α chains were isolated by gel filtration in SDS (7,10) (see Fig. 2A). Pro-α1 and pro-α2 chains were then separated by polyacrylamide gel electrophoresis in SDS (12). The ^{14}C-protein eluted from the gel slices was hydrolyzed in HCl and [^{14}C]hydroxyproline and [^{14}C]proline or [^{14}C]hydroxylysine and [^{14}C]lysine was determined using an amino acid analyzer column (12).

To synthesize [^{14}C]procollagen under aerobic conditions, 7 x 10^8 cells were incubated in 70 ml of medium for 180 min with either 20 μCi of [^{14}C]proline or 30 μCi of [^{14}C]lysine (10,11).

A: Ratio of [^{14}C]hydroxyproline to total [^{14}C]hydroxyproline plus [^{14}C]proline in pro-α chains from the medium. Pro-α1 (■) and pro-α2 (▲) chains synthesized under aerobic conditions; combined pro-α1 and pro-α2 chains (0---0) recovered after temporary anoxia.

B: Ratio of [^{14}C]hydroxylysine to total [^{14}C]hydroxylysine plus [^{14}C]lysine in pro-α1 chains from the medium of cells incubated under aerobic conditions (●——●) or under conditions of temporary anoxia (0---0).

The degree of under-hydroxylation of the procollagen secreted by the cells was even more marked if the cells were allowed to accumulate protocollagen at 37° as described above, and then the temperature of the cells was dropped to 31° or 34° just before they were exposed to air (Fig. 1A). In contrast, the degree of prolyl hydroxylation was the same at 31° to 40°, if the cells were continuously exposed to atmospheric 0_2 (Fig. 1A).

In the same type of experiments, using [^{14}C]lysine instead of [^{14}C]proline, the degree of hydroxylation of lysine residues in procollagen was also decreased if the cells were subjected to temporary anoxia (Fig. 1B). The ratio of [^{14}C]hydroxylysine to [^{14}C]hydroxylysine plus [^{14}C]lysine in the isolated pro-α1 chains was decreased regardless of the temperature at which the system was exposed to air, but the effect was more pronounced when the temperature was lowered. In contrast, there was no change in the degree of hydroxylation of the lysyl residues if the system was continuously exposed to 0_2 at temperatures varying from 31° to 40°. As indicated, the values for the pro-α1 chain are higher for the embryonic procollagen synthesized here than for chick collagen (see ref. 13).

Helical Stability of the Under-Hydroxylated Procollagen. Since the stability of the triple-helix formed by procollagen is directly dependent upon its hydroxyproline content (10,14-16), the results shown in Fig. 1 suggested that the procollagen secreted by cells subjected to anoxia and then exposed to air at 31° would be less stable than the procollagen synthesized at higher temperatures. To test this hypothesis,

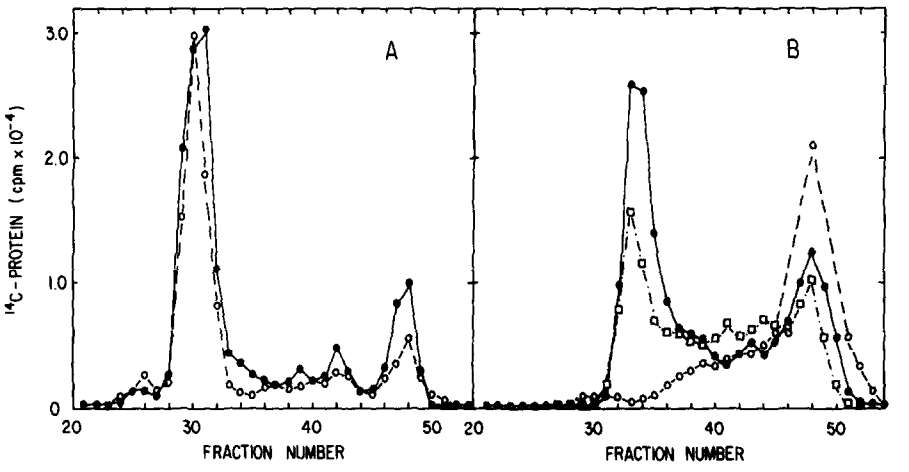

Figure 2. Helical stability of the [^{14}C]procollagen recovered from the medium. Tendon cells were incubated with [^{14}C]proline under conditions of temporary anoxia as described in Fig. 1. The medium ^{14}C-protein was then either directly treated with SDS-mercaptoethanol and chromatographed, or processed in the same manner after digestion with 300 µg of α-chymotrypsin per ml of medium at 30° or 36° for 3 hours (10). The void volume of the column was 48 ml (fraction 24) and the total volume was 132 ml (fraction 66). α-Chains of acid soluble calf skin collagen eluted in fractions 33-34.

A: Undigested medium ^{14}C-protein from cells exposed to O_2 at 37° (●——●) or at 31° (0---0).

B: Medium ^{14}C-protein after digestion with α-chymotrypsin. ^{14}C-Protein recovered after cells were exposed to O_2 at 37°; ^{14}C-protein was digested at 36° (●——●). ^{14}C-Protein recovered after cells were exposed to O_2 at 31°; ^{14}C-protein was digested either at 36° (0---0) or at 30° (□ -·-·- □).

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experiments similar to those described in Fig. 1 were carried out and the helical stability of the secreted [^{14}C]procollagen was tested by proteolysis with α-chymotrypsin under standardized conditions (see reference10). If the hydroxylation and secretion was allowed to proceed at 37°, most of the ^{14}C-protein in the medium was resistant to proteolysis at 36° and was recovered as α-chains (Fig. 2), indicating that the collagen portion of the [^{14}C]procollagen was triple-helical at this temperature. In contrast, if the hydroxylation of protocollagen and the subsequent secretion were allowed to

occur at 31°, the ^{14}C-protein in the medium was largely resistant to proteolysis at 30° but was digested into small peptides by proteolysis at 36°.

Rate of Secretion of Under-Hydroxylated Procollagen. In similar experiments the rate of secretion of under-hydroxylated but triple-helical procollagen was examined (Table I). The rate of secretion of [^{14}C]procollagen into the medium was the same whether the hydroxylation and secretion of the molecules occurred at 31° or 37° (Table I).

Table I. Secretion of under-hydroxylated procollagen after hydroxylation of intracellular protocollagen at 37° or 31°.

Tendon cells were labeled with [^{14}C]proline and subjected to temporary anoxia as described in Fig. 1. The samples were then exposed to atmospheric air at either 37° or 31° for 0 to 120 min. The total protein (cells plus medium) was 1.45×10^6 and 1.68×10^6 dpm, respectively.

Temperature for hydroxylation and secretion	^{14}C-Protein in medium (% of total)			
	0 min	15 min	60 min	120 min
37°	11.8	23.0	61.3	61.6
31°	12.8	17.3	57.2	62.9

Discussion. The presence of hydroxyproline markedly increases the thermal stability of the triple-helical structures formed by model synthetic peptides (14) or procollagen (15,16), apparently because the hydroxyl groups allow formation of additional H-bonds (17,18). Although intracellular protocollagen becomes triple-helical if cells containing it are cooled below 20°, extensive hydroxylation of prolyl residues is necessary for the triple-helix to be stable at 37° (7,10,15,16,19). Since folding of the pro-α chains into

the triple-helix prevents further hydroxylation by prolyl (20,21) and lysyl (22) hydroxylases, we initiated the experiments described here with the hypothesis that if the temperature of cells synthesizing procollagen were lowered, the polypeptides might become triple-helical at a lower than normal level of hydroxylation and the cells might synthesize and secrete a triple-helical but under-hydroxylated procollagen. This hypothesis was disproved by the observation that simply lowering the temperature to 34° or 31° had no effect on the degree of hydroxylation of the secreted procollagen. The results can probably be explained by the recent data (23,24) suggesting that in both embryonic tendon and cartilage cells exposed to O_2, helix formation is not limited by the synthesis of hydroxyproline. Previous studies in other cell systems (1,2,25) as well as more recent work with tendon cells (26) showed that if prolyl hydroxylase activity is not inhibited by restricting O_2 or iron, the hydroxylations of proline begin on nascent ribosomal chains. Although the hydroxylations continue until after final assembly of the polypeptides (26), they are apparently complete before the chain association necessary for helix formation can occur.

When the tendon cells employed here were incubated at 37° without O_2, they accumulated protocollagen comprised of pro-α chains which were already associated and linked by interchain disulfide bonds (23,24). In this circumstance, lowering the temperature to 31° or 34° before hydroxylation was initiated by exposing the system to air led to secretion of a triple-helical procollagen whose hydroxyproline content was less than two-thirds the control and whose hydroxylysine content was less than half of the control. Of special interest was

the observation that even when the hydroxylation of the accumulated protocollagen was allowed to proceed at 37°, the [^{14}C]procollagen subsequently secreted was under-hydroxylated by about 15% in terms of its hydroxyproline content and about 45% in terms of its hydroxylysine content.

Since the under-hydroxylated procollagen which was synthesized at 31° was secreted at the same rate as the more completely hydroxylated [^{14}C]procollagen synthesized at 37° (Table I), the results support previous indications that the triple-helical conformation (10,16), rather that the extent of hydroxylation or glycosylation, is the critical requirement for the molecule to be secreted at an optimal rate.

Since many connective tissues are relatively anaerobic under normal conditions and may be subject to temporary anoxia in pathological situations (27,28), the results presented here may have consequences for collagen synthesis by tendons and similar tissues in vivo. For example, temporary anoxia in such a tissue, particularly at the lowered temperatures likely to occur with ischemia, might well lead to the synthesis of a procollagen which would undergo rapid denaturation in vivo when the temperature of the tissue was subsequently increased by a few degrees. Also, the under-hydroxylation of lysyl residues produced by temporary anoxia in such tissues might well decrease the tensile strength of the collagen fibers formed in the tissues, since evidence from several sources suggests that cross-links of collagen derived from lysyl residues are less stable than those derived from hydroxylysine (29,30).

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