Effects of Temperature on Conformation, Hydroxylation, and Secretion of Chick Tendon Procollagen*

(Received for publication, September 21, 1973)

SERGIO A. JIMENEZ
From the Department of Medicine, School of Medicine, University of Pennsylvania, and Philadelphia General Hospital, Philadelphia, Pennsylvania 19104

MARGARET HARSH, LOIS MURPHY, AND JOEL ROSEN BLOOM†
From the Department of Biochemistry, Center for Oral Health Research, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19174

SUMMARY

When freshly isolated chick embryo tendon fibroblasts were incubated at 37° with [14C]proline and 0.5 mM α,α'-dipyridyl, a ferrous iron chelator which inhibits prolyl hydroxylase, the labeled unhydroxylated procollagen molecules accumulated intracellularly. When pepsin digestion at 15° was used as an enzymatic probe of conformation, these molecules were shown to be non-triple helical, indicating that hydroxylation of proline is necessary for triple helix formation at normal body temperatures. When cells which had accumulated unhydroxylated procollagen at 37° were then incubated at 25°, large amounts of unhydroxylated procollagen were secreted into the medium. Although these secreted molecules were sensitive to pepsin they were shown to be largely triple helical when prolyl hydroxylase was used as another probe of collagen conformation. The retained molecules were shown to be largely non-triple helical by this same test. This result suggests that triple helix formation may be necessary for normal secretion of procollagen by these cells.

When cells which had accumulated unhydroxylated procollagen at 37° were then incubated at different temperatures between 20° and 37° in the presence of excess ferrous iron to reverse the inhibition of prolyl hydroxylase, the accumulated molecules were secreted and the extent of hydroxyproline formation increased as the temperature during the reversal period increased. At 20° less than 1% of the [14C]proline in the secreted molecules was hydroxylated while at 37° this value increased to 33%. This result implies that under some conditions the body temperature of an organism may in part regulate the hydroxyproline content of the collagen molecules.

Recent studies from several laboratories have demonstrated that collagen is first synthesized as a precursor molecule called procollagen (1-4). The procollagen molecule is composed of three polypeptide chains which are larger than the α chains of interstitial collagen because of an extension present at the NH₂-terminus of each chain. These extensions are each approximately 25,000 daltons and differ from the α chain portion in their amino acid composition and conformation. The extensions are relatively poor in imino acids, relatively rich in acidic amino acids (5), and are susceptible to a number of proteolytic enzymes including pepsin (1-3). In contrast, the rest of the molecule is in a triple helical conformation, which is resistant to proteolysis by these enzymes and becomes susceptible to proteolysis only if the triple helical conformation is disrupted by heating or other means (6). Recent evidence indicates that the extensions present in the procollagen molecule are held together by disulfide bonds (7-9). The role of the extensions has not been clearly defined, but it has been postulated that they may serve to initiate triple helix formation by serving as registration peptides, they may facilitate secretion of collagen into the extracellular matrix, or they may control fibrillogenesis (10).

Although hydroxyproline occurs in all vertebrate interstitial collagens, it has been thought that it was not essential for the structural integrity of the collagen molecule, and its function has remained obscure (11). Recently we (12) and Berg and Prockop (13) have shown that hydroxyproline appears to stabilize the collagen triple helix. In further studies a series of procollagen samples, in which the hydroxyproline content varied from <1% to 44% of the total imino acids, was prepared from chick tendon fibroblasts, and the thermal stability of these procollagen preparations was then investigated by using pepsin digestion at different temperatures as an enzymatic probe of conformation (14). With the use of this technique, the denaturation temperature of the procollagen was found to be directly proportional to the hydroxyproline content. A denaturation temperature of 23.5° was found for the unhydroxylated procollagen and 37.9° for fully hydroxylated procollagen. These values agree well with measurements of denaturation temperatures made by optical rotation on unhydroxylated and fully hydroxylated collagen (13). These results also agree with the observation that the synthetic peptide (Pro-Hyp-Gly)₁₅ denatures at a significantly higher temperature than (Pro-Pro-Gly)₁₀ and that proline hydroxylation increases the thermal stability of the peptide α₁CB2...
obtained by CNBr cleavage of collagen from rat tail tendon and rat skin (15, 16).

Previous experiments with embryonic chick tibiae (17) and fibroblasts (18-21) indicated that when the hydroxylation of proline and lysine was inhibited, unhydroxylated collagen molecules were secreted at a markedly reduced rate and accumulated intracellularly. In the case of one type of fibroblast, it has been shown that the unhydroxylated molecules which were secreted were susceptible to pepsin at 15°C and, therefore, they were not entirely in a triple helical conformation (22). Since triple helix formation normally occurs intracellularly before collagen is secreted (23), it is possible that a reduction in hydroxyproline content could prevent such helix formation and cause a significant decrease in the rate of secretion.

In the present study we demonstrate that (a) intracellular unhydroxylated collagen is not triple helical at 37°C, which indicates that hydroxylation of proline is necessary for triple helix formation at normal body temperatures; (b) unhydroxylated procollagen molecules can be secreted by cells incubated at temperatures at which the molecules are largely triple helical, suggesting that triple helix formation may be required for normal secretion; and (c) when cells which have accumulated unhydroxylated procollagen molecules at 37°C are allowed to hydroxylate these molecules at different temperatures, then the degree of hydroxylation which can be achieved increases as the temperature increases. This result implies that under some conditions the body temperature of an organism may in part regulate the hydroxyproline content of the collagen molecules.

MATERIALS AND METHODS

Materials—[U-14C]Proline, 200 µCi per µmole, was purchased from New England Nuclear (Boston, Mass.). Crystalline pepsin, colcemide, cycloheximide, sodium dodecyl sulfate, and mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, Mo.). Reagents used for acrylamide disc gel electrophoresis and α,α′-dipyridyl were purchased from Eastman Kodak Co. (Rochester, N. Y.).

Isolation of Fibroblasts and Incubation Conditions—Fibroblasts were isolated from 17-day-old chick embryo tendons as previously described (24). Subsequently 106 cells per ml were incubated at 37°C in Krebs medium containing 2% fetal calf serum, 25 µg per ml of ascorbic acid, [14C]proline, and either 0.5 mM α,α′-dipyridyl or 1 µ M colcemide. The cells were incubated at the temperatures indicated and for the intervals shown for each experiment. In experiments in which it was desired to reverse the inhibition of prolyl hydroxylase by α,α′-dipyridyl, the cell suspensions were centrifuged and the cells resuspended in fresh media containing 1 mM FeSO4 and the incubations continued for the desired intervals. Further details of particular experiments are given in the tables and figures.

Treatment of Collagen Preparations with Pepsin—To determine whether the intracellular collagen synthesized by the cells under various experimental conditions was triple helical, limited pepsin digestion was used as an enzymatic probe of conformation (6, 12). To avoid changes in the conformation of the molecules, the samples were rapidly titrated to pH 2.0 with 1 N HCl and pepsin previously dissolved in 10 M HCl was added to a final concentration of 100 µg per ml. All manipulation of samples after termination of the incubations was carried out at room temperature as opposed to the usual temperature of 4°C in order to prevent possible reformation of triple helical molecules. The total elapsed time from the end of the incubation until the addition of pepsin was always less than 5 minutes. The pepsin digestion was performed at 15°C for 6 hours. In some experiments the duration of pepsin digestion was extended to 16 hours without any apparent change in results. At the end of the digestion, the solutions were neutralized, sodium dodecyl sulfate and mercaptoethanol were added to final concentrations of 1%, and the samples immediately placed in a boiling water bath for 3 min. The samples were then dialyzed against 0.01 M sodium phosphate, pH 7.0, 0.1% sodium dodecyl sulfate, and 0.1% mercaptoethanol and aliquots run on sodium dodecyl sulfate polyacrylamide gels or on 6% agarose columns.

Disc Gel Electrophoresis and Agarose Chromatography in Sodium Dodecyl Sulfate—Disc gel electrophoresis in sodium dodecyl sulfate was performed as described previously (12) with the use of 5% acrylamide gels and half the standard amount of cross linker. The gels were immediately chilled and cut into 1.5-mm fractions in order to minimize losses, and the fractions were solubilized with 30% H2O2 and counted in a scintillation counter. Duplicate gels were run containing radioactive samples and purified α and β rat tail collagen. These gels were stained with Coomassie blue and the radioactive peaks located relative to these markers. The positions of the radioactive peaks were identical in the stained and unstained gels when corrected for gel swelling during destaining. Radioactively labeled collagen samples were chromatographed on sodium dodecyl sulfate-agarose columns (Bio-Gel W, 200 to 400 mesh, Bio-Rad Laboratories, Rockville Centre, N. Y.) as described previously (3).

Preparation of Prolyl Hydroxylase and Unhydroxylated Procollagen—Prolyl hydroxylase was purified from 13 day old chick embryos as described by Halme et al. (25). The purification was carried through the DEAE-cellulose chromatography step, and the final enzyme preparation had a specific activity of 45 units per mg of protein when measured with 125 pg per ml of (Pro-Gly-Pro)3, molecular weight 2400, as substrate (purchased from Miles Laboratories, Inc., Kankakee, Ill.). One unit is defined as that amount of enzyme required to synthesize 1 µg of hydroxyproline in 1 hour at 37°C under conditions in which the concentrations of all other cofactors are saturating. Unhydroxylated [14C]procollagen was prepared by incubating isolated fibroblasts with 0.5 mM α,α′-dipyridyl for 5 hours and then extracting the [14C]procollagen from the cells with 0.5 M acetic acid at 4°C. The extract was centrifuged at 12,000 × g for 15 min, and the supernantant was then dialyzed exhaustively against 0.5 M acetic acid. This dialyzed material served as substrate for the hydroxylase. Conditions of the hydroxylation reaction are described in Table I.

RESULTS

Conformational State of Unhydroxylated Procollagen Retained Intracellularly at 37°C When the cells were incubated with [14C]proline and either 0.5 mM α,α′-dipyridyl or 1 µM colcemide for 2 hours, the procollagen synthesized by the cells was secreted at a markedly reduced rate relative to control cells, and the [14C]procollagen accumulated intracellularly. In the case of the α,α′-dipyridyl-treated cells, the [14C]procollagen contained essentially no [14C]hydroxyproline while the [14C]procollagen from the colcemide-treated cells was fully hydroxylated (28). In order to avoid changes in the conformation of the molecules, the cell suspensions were quickly centrifuged at the end of the incubation and aliquots of the resuspended cells immediately incubated with or without pepsin. The digest mixtures were neutralized and subjected to electrophoresis on sodium dodecyl sulfate polyacrylamide gels (Fig. 1). The fully hydroxylated collagen synthesized by the colcemide-treated cells was recovered as procollagen when pepsin was omitted from the digestion. After pepsin digestion, 85% of the radioactivity originally in the procollagen peak was recovered as α chains, thus indicating that it was predominantly triple helical. In contrast, less than 7% of the radioactivity originally in the unhydroxylated procollagen was recovered as α chains after pepsin digestion, and smaller molecular weight products were observed near the tracking dye. This result demonstrates that the unhydroxylated procollagen within the cells at 37°C was not triple helical.

It has been proposed that the additional amino acid residues at the NH2 termini, in which the chains are linked together by disulfide bonds, may serve as a nucleation site for triple helix formation. It was possible, therefore, that α,α′-dipyridyl could have interfered with the formation of these disulfide link-
Fibroblasts (10^6 per ml) were incubated with 3 μCi per ml of [14C]proline and 0.5 mM α,α'-dipyridyl in Krebs medium for 1 hour at 37°. The cell suspension was then centrifuged at 1500 × g for 2 min and the cells resuspended at 10^6 per ml in a modified Krebs medium containing 1 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, pH 7.4, in place of sodium phosphate buffer, 7 mM KCl in place of KH_2PO_4, 0.1 mM α,α'-dipyridyl, and 25 μg per ml of catalase (Sigma Chemical Co.), 0.5 mM FeSO_4, 0.5 mM ethanesulfonic acid, pH 7.8. Unhydroxylated procollagen which was added at the end of the incubation (see below). Aliquots of the cells were then incubated at 25° or 37° for 3 hours and the suspensions then centrifuged. The cells were resuspended in an equal volume of fresh N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid medium, sonicated briefly at 25°, and 1-ml aliquots of the supernatants and cells incubated at 25° or 37° for 1 hour in a 2-ml reaction mixture, each ml of which contained 4 units of procollagen, 1 mg of bovine serum albumin, 0.5 mg of catalase (Sigma Chemical Co.), 0.5 mM FeSO_4, 0.5 mM α-ketoglutarate, 0.1 mM dithiothreitol, 2 mM ascorbate, and 50 mM Tris-HCl buffer, pH 7.8. Unhydroxylated procollagen which had been denatured by heating to 45° for 45 min in the N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid-Krebs medium immediately prior to incubation with the enzyme was used as a control. The incubations were terminated by addition of 2 ml of 12 M HCl, and the mixtures were then hydrolyzed at 108° for 24 hours. The [14C]hydroxyproline content of the samples was then assayed by a specific chemical procedure (27). The values in the table represent the average of duplicate incubations.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydroxylation temperature</th>
<th>25°</th>
<th>37°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total dpm X 10^{-4}</td>
<td>Degree of hydroxylation</td>
<td>Total dpm X 10^{-4}</td>
</tr>
<tr>
<td>Denatured unhydroxylated procollagen</td>
<td>36.1</td>
<td>32.3</td>
<td>35.7</td>
</tr>
<tr>
<td>Cell incubation (25°)</td>
<td>91.4</td>
<td>5.2</td>
<td>91.7</td>
</tr>
<tr>
<td>Secreted</td>
<td>97.2</td>
<td>13.7</td>
<td>96.5</td>
</tr>
<tr>
<td>Intracellular</td>
<td>45.5</td>
<td>7.2</td>
<td>53.4</td>
</tr>
<tr>
<td>Cell incubation (37°)</td>
<td>97.6</td>
<td>20.1</td>
<td>102.1</td>
</tr>
<tr>
<td>Secreted</td>
<td>177.9</td>
<td>0.2</td>
<td>177.9</td>
</tr>
</tbody>
</table>

Values are 100 × [14C]hydroxyproline per total 14C.
Prepared as described under “Materials and Methods.” The degree of hydroxylation was <1%.

ages and in this way impaired triple helix formation. Aliquots of cells incubated as described above were therefore prepared for gel electrophoresis in sodium dodecyl sulfate containing no mercaptoethanol and 0.1 M iodoacetamide to scavenge any possible reducing compounds present in the sodium dodecyl sulfate itself (29). The results shown in Fig. 2 demonstrate that when reducing agents were absent only about 25% of the unhydroxylated and 18% of the fully hydroxylated intracellular procollagen was found as single chains, indicating that α,α'-dipyridyl did not interfere with disulfide formation.

Secretion of Unhydroxylated Procollagen at 25°—Recent experiments using prolyl hydroxylase have indicated that unhydroxylated procollagen is largely in a triple helical conformation only at or below 25° at neutral pH (30, 31). We therefore incubated cells, containing accumulated unhydroxylated procollagen, at 25° or 37° in order to compare the rate at which they secreted the unhydroxylated molecules at the two temperatures (Fig. 3). When the cells were incubated at 25° there was a progressive secretion of the accumulated unhydroxylated molecules so that after 3 hours 41% of the nondialyzable radioactivity was found in the medium (Fig. 3). When the cells were incubated at 37°, the secretion was irregular, and only 26% was found in the medium. Corresponding to the increase in radioactivity in the medium at 25°, there was a decrease in the radioactivity found intracellularly. At 37° the secreted radioactivity was slightly less than the corresponding intracellular decrease.

These results suggested that differences might exist between the structure of the protein secreted at 25° and 37°, and aliquots of the secreted proteins were, therefore, subjected to electrophoresis on polyacrylamide gels. The results (Fig. 4) demonstrated that over 60% of the radioactivity in the proteins secreted at 25° was recovered in the molecular weight range of 125,000. However, when the secreted proteins were digested with pepcid at 15° before electrophoresis, only lower molecular weight peptides were recovered. This result indicates that although the chains had a molecular weight equal to that of procollagen, they were either completely denatured or contained regions of local

---

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydroxylation temperature</th>
<th>25°</th>
<th>37°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total dpm X 10^{-4}</td>
<td>Degree of hydroxylation</td>
<td>Total dpm X 10^{-4}</td>
</tr>
<tr>
<td>Denatured unhydroxylated procollagen</td>
<td>36.1</td>
<td>32.3</td>
<td>35.7</td>
</tr>
<tr>
<td>Cell incubation (25°)</td>
<td>91.4</td>
<td>5.2</td>
<td>91.7</td>
</tr>
<tr>
<td>Secreted</td>
<td>97.2</td>
<td>13.7</td>
<td>96.5</td>
</tr>
<tr>
<td>Intracellular</td>
<td>45.5</td>
<td>7.2</td>
<td>53.4</td>
</tr>
<tr>
<td>Cell incubation (37°)</td>
<td>97.6</td>
<td>20.1</td>
<td>102.1</td>
</tr>
<tr>
<td>Secreted</td>
<td>177.9</td>
<td>0.2</td>
<td>177.9</td>
</tr>
</tbody>
</table>

Values are 100 × [14C]hydroxyproline per total 14C.
Prepared as described under “Materials and Methods.” The degree of hydroxylation was <1%.

---

**Notes**

- α Values are 100 × [14C]hydroxyproline per total 14C.
- b Prepared as described under “Materials and Methods.” The degree of hydroxylation was <1%.

---

**Fig. 1.** Polyacrylamide sodium dodecyl sulfate gel electrophoresis of intracellular procollagen after incubation with or without peptic. Aliquots of cells which had been incubated with 1 μCi of [14C]proline and 0.5 mM α,α'-dipyridyl, B, and 14C-proline for 2 hours were then incubated with or without 100 μg per ml of peptic at pH 2.0 for 6 hours at 15°. At the end of the digestion the solutions were neutralized, prepared for electrophoresis, run on 5% polyacrylamide gels, and the gels fractionated and counted as described under “Materials and Methods.” •—•, incubation with peptic; O——O, without peptic; f, rat tail β chains; r, rat tail α chains; s, tracking dye.
4483

Fig. 2. Polyacrylamide sodium dodecyl sulfate gel electro-
phoresis of intracellular procollagen prepared for electrophoresis in the presence or absence of reducing agent. Aliquots of cells which had been incubated with 0.5 mM α,α′-dipyridyl, A, or 1 μM colcemide, B, were resuspended in 0.01 M sodium phosphate, pH 7.0, 1% sodium dodecyl sulfate containing either 1% mercapto-
ethanol or 0.1 M iodoacetamide, and then placed in a boiling water bath for 2 min. The samples were then dialyzed against 0.01 M sodium phosphate, pH 7.0, 0.1% sodium dodecyl sulfate contain-
ing either 0.1% mercaptoethanol or 0.1 M iodoacetamide. Elec-
trophoresis was then carried out as described in Fig. 1 and under “Materials and Methods,” except that it was continued for 1 hour longer in order to allow the higher molecular weight mole-
cules in the nonreduced samples to enter the gel further.

O-O, prepared for electrophoresis with 1% mercaptoethanol; O-O, prepared with 0.1 M iodoacetamide.

denaturation, sensitive to pepsin, which led to degradation of the collagen. Similar digestion of the intracellular proteins demon-
strated that they were also sensitive to pepsin. Our findings with the proteins secreted at 37° agreed with previous results showing that only degraded fragments were found in the medium (21).

Ability of Unhydroxylated Procollagen Isolated from Cells Incu-
bated at 25° or 37° to Serve as Substrate for Prolyl Hydroxylase— Previous experiments have indicated that prolyl hydroxylase can only hydroxylate regions of the collagen molecule which are not triple helical (30, 31). We, therefore, tested the ability of the unhydroxylated proteins isolated from the medium and cells incubated at 25° or 37° to serve as substrate for prolyl hydroxyl-
ase (Table I). The proteins secreted at 25° had a degree of hydroxylation of 5.9% after incubation with excess prolyl hydroxy-
lase at 25°, and this value increased to 19.9% when the hydroxylation was carried out at 37°. In contrast, the proteins secreted at 37° were relatively poor substrates and had a degree of hydroxylation of 7.2% when hydroxylated at 25° and 11.1% when hydroxylated at 37°. The proteins retained intracellularly at 25° or 37° also demonstrated a significant difference in their ability to serve as substrate at the two hydroxylation tempera-
tures of 25° and 37°. The proteins in the cells incubated at 25° were hydroxylated to about 14 to 15%, at both hydroxylation temperatures. In contrast, the proteins in the cells incubated at 37° were hydroxylated to 20.1% at 25° and to 30.7% at 37°. We have interpreted these findings to mean that molecules which are largely triple helical are secreted at 25° and that molecules which are largely non-triple helical are retained intracellularly at both 25° and 37°. These results are considered more exten-
sively under “Discussion,” but they suggest that triple helicity may be required for normal secretion of procollagen.

Intracellular Hydroxylation at Different Temperatures of Un-
hydroxylated Procollagen Accumulated at 25°—When extracted unhydroxylated procollagen or collagen was incubated with prolyl hydroxylase at various temperatures, the amount of hydroxyproline which could be formed increased sharply between 26° and 30° (30, 31). In order to determine whether temperature had a similar effect on the intracellular hydroxylation of accumu-

Fig. 3. Secretion of unhydroxylated collagen at 25° and 37°. Cells were incubated for 1 hour at 37° with 0.5 mM α,α′-dipyridyl as described under “Materials and Methods.” Cycloheximide was then added to a final concentration of 25 μg per ml and aliquots of the cells incubated at 25° and 37°. At hourly intervals 1-ml aliquots were removed and the suspensions centrifuged at 1200 × g for 2 min. The cells were then resuspended in 1 ml of 0.01 M sodium phosphate buffer, pH 7.4, containing 1% sodium dodecyl sulfate and 1% mercaptoethanol. Sodium dodecyl sulfate and mercaptoethanol were added to a final concentration of 1% to the supernatants. The cell suspensions and superna-
танты were then placed in a boiling water bath for 2 min and then dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, 0.1% sodium dodecyl sulfate, and 0.1% mercaptoethanol. Total radio-
activity was then determined in the dialyzed samples. Zero time in the graphs is after the initial 1-hour incubation at 37°. A, supernatant; B, cells; O-O, 37°; •, 25°.
labeled procollagen independently of the effect of temperature on the rate of protein synthesis, the following experiments were performed. Unhydroxylated procollagen was accumulated intracellularly at 37° by incubating cells with 0.5 mM \(\alpha_1\alpha_2\)-dipryridyl for 1 hour. The cells were then washed and resuspended in fresh medium containing 25 \(\mu\)g per ml of cycloheximide to stop protein synthesis and 1 mM FeSO\(_4\) to reverse the inhibition of prolyl hydroxylase. Aliquots of the cell suspension were then incubated at 20°, 25°, 30°, or 37° for 3 hours. At hourly intervals 1-ml aliquots were taken, the cells separated by centrifugation, and the resuspended cells and supernatants dialyzed separately against running tap water. The dialyzed samples were then hydrolyzed and assayed for total radioactivity and \(^{14}\)C-hydroxyproline content. Zero time in this experiment is after the initial 1-hour incubation at 37°.

### Table II

Kinetics of intracellular hydroxylation at different temperatures of procollagen accumulated at 37°

<table>
<thead>
<tr>
<th>Cell incubation temperature</th>
<th>Intracellular %</th>
<th>Secreted %</th>
<th>Secreted Intracellular + Secreted %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero time</td>
<td>17.6 ± 0.5</td>
<td>9.4 ± 0.6</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>20°</td>
<td>21.9 ± 0.4</td>
<td>3.9 ± 0.4</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>2 hours</td>
<td>20.6 ± 1.8</td>
<td>4.3 ± 0.4</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>3 hours</td>
<td>19.6 ± 2.3</td>
<td>4.7 ± 0.5</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>25°</td>
<td>20.0 ± 2.6</td>
<td>3.8 ± 0.3</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>1 hour</td>
<td>19.3 ± 4.2</td>
<td>4.4 ± 0.6</td>
<td>3.7 ± 0.7</td>
</tr>
<tr>
<td>3 hours</td>
<td>15.2 ± 5.1</td>
<td>5.1 ± 1.2</td>
<td>4.1 ± 1.2</td>
</tr>
<tr>
<td>30°</td>
<td>20.3 ± 8.3</td>
<td>4.2 ± 1.6</td>
<td>7.2 ± 1.6</td>
</tr>
<tr>
<td>2 hours</td>
<td>15.7 ± 9.6</td>
<td>5.8 ± 5.1</td>
<td>8.4 ± 5.1</td>
</tr>
<tr>
<td>3 hours</td>
<td>17.3 ± 8.0</td>
<td>6.9 ± 7.2</td>
<td>7.8 ± 7.2</td>
</tr>
<tr>
<td>37°</td>
<td>19.8 ± 14.2</td>
<td>5.7 ± 8.3</td>
<td>13.4 ± 8.3</td>
</tr>
<tr>
<td>1 hour</td>
<td>17.7 ± 15.5</td>
<td>7.3 ± 10.1</td>
<td>14.4 ± 10.1</td>
</tr>
<tr>
<td>3 hours</td>
<td>15.6 ± 15.6</td>
<td>8.7 ± 10.0</td>
<td>13.6 ± 10.6</td>
</tr>
</tbody>
</table>

* Values are 100 × \(^{14}\)C-hydroxyproline per total \(^{14}\)C.

**DISCUSSION**

It was previously reported (21) that unhydroxylated procollagen which was retained intracellularly was triple helical since it was resistant to pepsin digestion at 15° and formed normal segment-long spacing aggregates at 4°. However, more recent experiments (12-16), which demonstrated that hydroxyproline is crucial to the thermal stability of collagen and procollagen, suggested that unhydroxylated procollagen might not be triple helical within the cell at 37°. In the present experiments the conformation of the intracellular unhydroxylated procollagen was examined by pepsin digestion under conditions designed to mini-
mize the opportunity for conformational changes in the protein. Under these conditions 93% of the unhydroxylated molecules were digested while only 15% of the hydroxylated intracellular molecules were digested. By this test then the unhydroxylated molecules were largely not triple helical while the hydroxylated molecules were triple helical. Since most of both the unhydroxylated and hydroxylated chains appeared to be held together by disulfide linkages (Fig. 2), the difference in the conformation of the two types of molecules cannot be due to prevention of disulfide bond formation by \( \alpha, \alpha'- \)dipyridyl. In the earlier report (21) the unhydroxylated collagen was extracted from the cells and apparently reformed native molecules during the extraction and prolonged dialysis period at 4°.

Experiments using fibroblasts in culture in which hydroxyproline formation was inhibited by incubation in ascorbate-deficient media (20, 22, 32) or through Fe chelation (18) have indicated that markedly underhydroxylated molecules can be secreted although probably at a reduced rate relative to the secretion of fully hydroxylated molecules. Experiments using freshly isolated tendon fibroblasts showed that total inhibition of hydroxylation prevented secretion of whole procollagen molecules at 37°. The reason for the difference between the cultured fibroblasts and the freshly isolated fibroblasts is not clear. Perhaps the secretory ability of the freshly isolated cells may have been impaired by the proteolytic digestion required to free them from the extracellular matrix, or alternatively the ability of the fibroblasts in culture to discriminate triple helical from non-helical molecules may be impaired. The present results indicate that the inability of the isolated tendon cells to secrete unhydroxylated procollagen can be overcome by incubating the cells at 25°, since large amounts of unhydroxylated procollagen were secreted at 25° and essentially none at 37°. This is all the more remarkable since we would expect all cellular functions including secretory ability to be considerably slower at 25° than at 37°.

The unhydroxylated molecules secreted at 25° were not perfectly triple helical, however, since they were sensitive to pepsin. Pepsin digestion, as presently used, is a stringent test of triple helicity since a single disruption of the triple helix probably leads to digestion. Recent experiments studying the ability of unhydroxylated procollagen to serve as a substrate for prolyl hydroxylase as a function of temperature have strongly indicated that only procollagen molecules which are not in a triple helical conformation can be hydroxylated (30, 31). We therefore used prolyl hydroxylase as another probe of collagen conformation. The results with the secreted unhydroxylated proteins were fairly clear. The unhydroxylated procollagen secreted at 25° was hydroxylated to a much greater extent when incubated with prolyl hydroxylase at 37° than at 25°. This result suggested that large regions of the molecules were triple helical at 25°. Control experiments using denatured procollagen proved that completely denatured molecules could be fully hydroxylated at 25°. The proteins found in the medium at 37° were relatively poor substrates at both hydroxylation temperatures of 25° and 37°, probably because these proteins were either non-collagenous or degraded unhydroxylated collagen. Since the molecules retained

---

**Table III**

Degree of hydroxylation of procollagen hydroxylated intracellularly at different temperatures

The five peak fractions containing the procollagen in each of the four chromatograms illustrated in Fig. 5 were pooled and an equal volume of 12 N HCl added. The samples were hydrolyzed for 24 hours at 108° and analyzed for their [\(^{14}C\)]hydroxyproline content. Pooled peak fractions from similar chromatograms of the intracellular labeled proteins were also analyzed.

<table>
<thead>
<tr>
<th>Temperature of hydroxylation</th>
<th>Intracellular</th>
<th>Secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak total cpm ( \times 10^3 )</td>
<td>Degree of hydroxylation</td>
</tr>
<tr>
<td>20°</td>
<td>39.4</td>
<td>25.9</td>
</tr>
<tr>
<td>25°</td>
<td>54.3</td>
<td>20.0</td>
</tr>
<tr>
<td>30°</td>
<td>47.5</td>
<td>10.6</td>
</tr>
<tr>
<td>37°</td>
<td>55.7</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Values are 100 \( \times \)[\(^{14}C\)]hydroxyproline per total \(^{14}C\).*
intradcellularly at 25° were equally good substrates for hydroxylation at 25° and 37°, they appeared to be less triple helical than the secreted ones. Although the findings with the molecules retained at 37° qualitatively agree with the interpretation that they were largely non-triple helical, one quantitative inconsistency exists. That is the fact that these molecules were somewhat better substrates at 37° than at 25°. We have no certain explanation for this observation, but it is possible that limited helical regions in fact existed intracellularly or were formed either during the work up of the cells at room temperature prior to the hydroxylation at 25° or during the hydroxylation at 25° itself. Nevertheless, by the criterion of their ability to be hydroxylated, the secreted procollagen molecules were largely triple helical, and the retained intracellular molecules were largely non-helical, and it is possible that the proper conformation may be essential for rapid secretion of procollagen by these cells.

When unhydroxylated procollagen was incubated with purified prolyl hydroxylase in a cell-free system at different temperatures, the amount of hydroxyproline which could be formed increased sharply between 20° and 30°, providing the substrate was not denatured prior to incubation with the enzyme (30, 31). These experiments suggested that under certain conditions temperature might regulate the degree of hydroxylation achievable intracellularly. The present experiments, in which the process of protein synthesis has been dissociated from hydroxylation, and the accumulated unhydroxylated procollagen then hydroxylated intracellularly by reversal of inhibition of prolyl hydroxylase at different temperatures support the possibility that such regulation can take place. Kinetic experiments (Table II) at the different temperatures showed that the hydroxylation reaction was essentially complete 2 hours after the inhibition of prolyl hydroxylase was reversed. Hydroxyproline determinations on the isolated procollagen (Table III) showed that the degree of hydroxylation of the molecules increased markedly when the temperature increased during the reversal period. One possible explanation of these results is that partial triple helix formation occurs more readily at lower temperatures, and the triple helical regions of the molecule cannot be hydroxylated further, resulting in lower degrees of hydroxylation. These experiments suggest that under some conditions the body temperature of an organism may in part regulate the hydroxyproline content of collagen molecules.

Acknowledgments—We thank Miss Sandy Schnee and Mrs. Marie Pratt for superior technical assistance.

REFERENCES

Effects of Temperature on Conformation, Hydroxylation, and Secretion of Chick Tendon Procollagen

Sergio A. Jimenez, Margaret Harsch, Lois Murphy and Joel Rosenbloom


Access the most updated version of this article at http://www.jbc.org/content/249/14/4480

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/249/14/4480.full.html#ref-list-1