THE THERMAL TRANSITION OF A NON-HYDROXYLATED FORM OF COLLAGEN. EVIDENCE FOR A ROLE FOR HYDROXYPROLINE IN STABILIZING THE TRIPLE-HELIX OF COLLAGEN

Richard A. Berg and Darwin J. Prockop

Department of Biochemistry, The Rutgers Medical School,
College of Medicine and Dentistry of New Jersey,
New Brunswick, New Jersey, 08903

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Summary: Protocollagen, a non-hydroxylated form of collagen, was extracted with cold 0.1 N acetic acid from embryonic tendon cells incubated with α,α′-dipyridyl and the protein was purified by controlled proteolytic digestion. The resulting modified protocollagen was shown to consist of polypeptides the same size as α1 and α2 chains of collagen and had a thermal transition by optical rotation similar to collagen. The T_m however was 24°, a value which was 15° lower than the T_m of an hydroxylated form of collagen from the same source. The results suggest that hydroxylated proline increases the thermal stability of collagen.

Protocollagen has recently been extracted from matrix-free cells from chick embryos (1) incubated with the iron chelator α,α′-dipyridyl so that the prolyl and lysyl hydroxylases are inhibited and protocollagen accumulates intracellularly (2). Protocollagen extracted from the cells with cold 0.1 N acetic acid was comprised of polypeptide chains similar to the pro-α chains of the precursor form of collagen known as procollagen (3) except that it contained less than two residues of hydroxyproline and less than one residue of hydroxylysine per 1,000 amino acid residues (2). Recently, we have found that by treating the protocollagen preparation with α-chymotrypsin at 15° under conditions in which the helical portion of collagen is not digested, it is possible to purify several hundred micrograms of triple-helical protocollagen

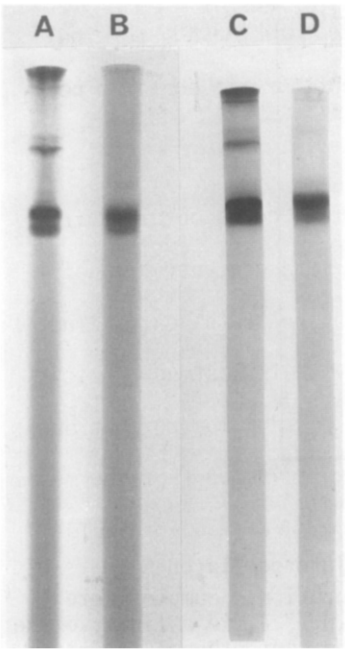
Abbreviations: Protocollagen-C, modified protocollagen which was first extracted with cold acetic acid to allow the polypeptides to become helical and then subjected to limited digestion with α-chymotrypsin (see Materials and Methods); procollagen-C, modified procollagen prepared from the medium of matrix-free tendon cells and subjected to limited digestion with α-chymotrypsin; SDS, sodium dodecyl sulfate.

C, a modified form of protocollagen which does not contain the NH_2-terminal extensions and is therefore similar to collagen except that it contains no hydroxylated proline or lysine (4).

MATERIALS AND METHODS. About 1.5 x 10^9 matrix-free cells obtained from the tendons of 120 17-day-old embryos were incubated at a concentration of 7.5 x 10^6 cells/ml of modified Krebs medium containing 10% fetal calf serum (1). After pre-incubation for 35 min with 0.3 mM α,α´-dipyridyl (Eastman Chemical Co.) with shaking at 37°, 10 μCi of [^{14}C]proline was added prior to continuing incubation for an additional 4 hours. The cells were separated from the medium, frozen at minus 20° and homogenized in 20 ml of ice cold 0.1 M acetic acid with a Teflon and glass homogenizer. The homogenate was dialyzed against 0.1 M acetic acid at 4° for 12 hours, centrifuged at 20,000 x g for 30 min, and dialyzed against 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.4 at 4°. The supernatant protein was precipitated with 176 mg/ml of ammonium sulfate (Baker Chemical Co.) (2). To prepare [^{14}C]protocollagen-C, the ammonium sulfate precipitate was suspended in 10 ml of the NaCl-Tris buffer, and then 300 μg/ml of α-chymotrypsin (46 units/mg; Worthington) was added. The sample was dialyzed against the NaCl-Tris buffer at 4° for 15 hours and then at 15° for 6 hours. The [^{14}C]protocollagen-C was precipitated with 176 mg/ml of ammonium sulfate, dialyzed against 0.1 M acetic acid, and stored at 4°.

[^{14}C]Procollagen was prepared from the proteins secreted in the medium by the cells incubated for 6 hours under the same conditions but without α,α´-dipyridyl and without fetal calf serum (1). The medium was dialyzed against the NaCl-Tris buffer at 4° and precipitated with 176 mg/ml ammonium sulfate. [^{14}C]Procollagen-C (4) was prepared by digestion of the precipitate with 100 μg/ml of α-chymotrypsin as described above.

RESULTS AND DISCUSSION. The [^{14}C]protocollagen-C and [^{14}C]procollagen-C were examined by polyacrylamide gel electrophoresis in SDS (6) and with both samples essentially all the protein was recovered in two bands which had



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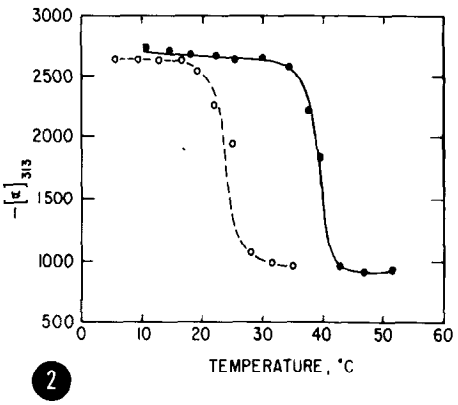

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Fig. 1. Polyacrylamide gel electrophoresis in SDS of [^{14}C]procollagen-C and [^{14}C]protocollagen-C was carried out according to the method of Weber and Osborn (6) with 5% acrylamide gels and the gels were stained with Coomassie Brilliant Blue. Neutral salt soluble collagen was prepared from the skin of 20-day lathyritic chick embryos (12). About 10 µg of protein were applied to each gel and the gels were run until the dye front of Bromphenol Blue reached the anodal end. Gel A, neutral salt soluble collagen, and gel B, [^{14}C]protocollagen-C from one electrophoretic experiment. Gel C, neutral salt soluble collagen, and gel D, [^{14}C]procollagen-C from a second experiment.

Fig. 2. Thermal transition by optical rotation of [^{14}C]protocollagen-C (0----0) and [^{14}C]procollagen-C (●——●) in 0.1 N acetic acid. Optical rotation was measured in a Cary model 60 recording spectropolarimeter at 313 nm with a 1 cm water-jacketed cell. The samples were equilibrated at each new temperature for 35 to 40 min and the protein concentration was determined by amino acid analysis after melting. The concentration of [^{14}C]protocollagen-C was 196 µg/ml and of [^{14}C]procollagen-C was 242 µg/ml. The samples were centrifuged at 30,000 x g for 45 min just before being placed in the cell.

mobilities similar to the α1 and α2 chains of collagen (Fig. 1). Also, when the gels were cut into 1.5 cm slices and hydrolyzed, essentially all of the ^{14}C was recovered in the two bands (4). The results indicated therefore that the [^{14}C]protocollagen-C and [^{14}C]procollagen-C were highly purified and were comprised of polypeptides of the same size as the α1 and α2 chains of collagen. As reported elsewhere (4), the amino acid compositions of the samples were essentially the same as collagen except that [^{14}C]protocollagen-C contained

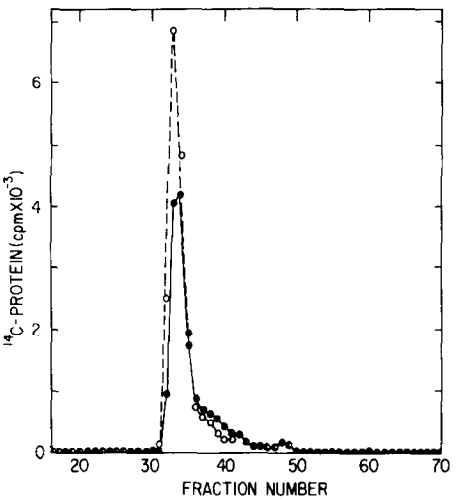

Fig. 3. Gel filtration in SDS-agarose of [^{14}C]protocollagen-C (0----0) and [^{14}C]procollagen-C (●——●) after thermal denaturation. Samples were adjusted to 0.1% of SDS and 0.1 M sodium phosphate, pH 7.4, in a final volume of 2 ml. They were heated at 100° for 5 min and 2-mercaptoethanol (Aldrich Chemical Co.) was added to a final concentration of 1% for further incubation at 37° for 4 hours. The samples were then directly applied to an SDS-agarose column (Bio-Gel, A-5m, 200-400 mesh, Bio-Rad) which was 1.9 x 80 cm (5). The fractions were 2.0 ml and the cpm observed in 0.3 ml aliquots are indicated. The total volume was 136 ml (fraction 68) and the void volume was 48 ml (fraction 24). Collagen α chains eluted with a peak in fraction 33 to 34 and pro-α chains from tendon cells (1) eluted in fraction 30 to 31.

less than one residue per 1,000 of hydroxyproline and hydroxylysine, and it was correspondingly rich in proline and lysine.

In examining the helical stability of [^{14}C]protocollagen-C, [^{14}C]procollagen was used as control for an hydroxylated form of the same protein. [^{14}C]Procollagen-C was found to have a sharp thermal transition with a T_m of 39° (Fig. 2), a value comparable to those reported for collagens from other tissues of the chick embryo (7). Under the same conditions [^{14}C]protocollagen-C had a thermal transition similar to that of the [^{14}C]-procollagen-C (Fig. 2). However, the temperature for the transition was lower. The T_m was 24° in the experiment shown (Fig. 2) and 26° in a separate experiment.

Since both the [^{14}C]protocollagen-C and the [^{14}C]procollagen-C were purified by limited digestion with α-chymotrypsin, it was essential to

establish that there was no proteolysis during measurement of the thermal transition. There was no evidence by amino acid analysis (4) or polyacrylamide gel electrophoresis (Fig. 1) that the samples contained α-chymotrypsin. More importantly, there was no evidence of degradation of either sample by α-chymotryptic activity when the melted samples were re-examined by polyacrylamide gel electrophoresis in SDS (see Fig. 1) or by gel filtration on SDS-agarose (Fig. 3).

The [^{14}C]protocollagen-C and [^{14}C]procollagen-C were the same in chemical composition except for the absence of hydroxyproline, hydroxylysine and glycosylated hydroxylysine in the [^{14}C]protocollagen-C. Since the hydroxylysine content of most collagens is small, and since collagens with four-fold differences in hydroxylysine contents have similar T_m values (7), it seems unlikely that hydroxylysine contributes significantly to the stability of the collagen triple-helix. The large difference in T_m between [^{14}C]protocollagen-C and [^{14}C]procollagen-C provides evidence therefore for a role for hydroxyproline in stabilizing the triple-helical conformation of collagen*. This conclusion is consistent with recent observations by Sakakibara, et al. (10) indicating that hydroxyproline stabilizes the collagen-like helix formed by synthetic polypeptides such as $(Pro\text{-}Hyp\text{-}Gly)_{10}$.

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* For previous suggestions of a possible role for hydroxyproline in the structure of collagen see references 8, 9, and 10.

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