Purification of [^{14}C]Protocollagen and Its Hydroxylation by Prolyl-Hydroxylase†

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ABSTRACT: Protocollagen, the unhydroxylated precursor form of collagen, was purified from matrix-free cells of embryonic chick tendon and examined for its ability to serve as a substrate for prolyl-hydroxylase. The cells were incubated in the presence of [^{14}C]proline and 0.3 mM α,α'-dipyridyl, an inhibitor of prolyl-hydroxylase, and [^{14}C]-protocollagen was purified by extraction in 0.1 N acetic acid, ammonium sulfate precipitation, and limited proteolytic digestion. The enzymic digestion with either pepsin or α-chymotrypsin was carried out at 15° under conditions in which the NH_2-terminal extensions on the [^{14}C]protocollagen were removed and contaminating proteins were digested but the helical portion of the molecule remained intact. The enzyme-modified protocollagen was pure by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and was comprised of polypeptide chains which were similar to the α1 and α2 chains of collagen except that they contained no hydroxylated proline or lysine. The enzyme-modified [^{14}C]protocollagen served as a substrate for the synthesis of hydroxyproline by prolyl-hydroxylase at 25, 30, or 37° but not at 15 or 20°. There was a sharp change in the ability of the enzyme-modified [^{14}C]protocollagen to serve as a substrate for prolyl hydroxylase at about 28°, a temperature close to the T_m for the thermal transition of enzyme-modified protocollagen. If the enzyme-modified protocollagen was first heat denatured, it was readily hydroxylated by prolyl hydroxylase at 15°. The results demonstrated that hydroxylation of prolyl residues by prolyl-hydroxylase was dependent upon the structure of the prolyl-containing substrate. Since ultracentrifugation indicated that the substrate was not grossly aggregated at 15° and neutral pH, the results suggested that a helical conformation of the substrate in itself prevented its hydroxylation. The K_m for the hydroxylation of the random-coil form of unmodified [^{14}C]protocollagen at 37° was found to be 2 nM and the turnover number for prolyl-hydroxylase expressed on the basis of moles of hydroxy[^{14}C]proline per mole of enzyme was 4 sec^{-1}.

Hydroxyproline in collagen is synthesized by the hydroxylation of proline after it is incorporated into peptide linkages (for review, see Grant and Prockop, 1972). Recently, matrix-free cells from tendons of chick embryos were found to synthesize collagen at a rapid rate *in vitro* (Dehm and Prockop, 1971, 1972) and with this system it was possible to isolate protocollagen, the proline- and lysine-rich polypeptide precursor of collagen which accumulates intracellularly when the prolyl- and lysyl-hydroxylases are inhibited with an iron chelator such as α,α'-dipyridyl or with anaerobic conditions. Protocollagen isolated from the cells by prolonged extraction with cold acetic acid was shown to be in a helical conformation similar to collagen in that it was largely resistant to digestion by pepsin at 15° (Jimenez *et al.*, 1973). The protocollagen preparation contained less than two residues of hydroxyproline per 1000 residues, and at 37° it served as a substrate for the synthesis of hydroxyproline by prolyl-hydroxylase. Also, it was shown that the protocollagen was comprised of polypeptides which were the same size as the pro-α chains of the presursor form of collagen known as "procollagen" (Bellamy and Bornstein, 1971; Stark *et al.*, 1971; Vuust and Piez, 1972) or "transport form" (Layman *et al.*, 1971; Jimenez *et al.*, 1971; Dehm *et al.*, 1972). After digestion of the protocollagen at 15° with pepsin, a procedure which removes the NH_2-terminal extensions of procollagen, the individual polypeptide chains were reduced to the same size as α chains of collagen (Jimenez *et al.*, 1971, 1973).

We have now found that by extracting a relatively large number of matrix-free tendon cells with cold 0.1 N acetic acid and treating the extracts with pepsin or α-chymotrypsin at 15°, it is possible to isolate several hundred micrograms of purified, enzyme-modified protocollagen. Recent studies on the optical rotation of the purified, enzyme-modified protocollagen showed that it had a temperature-dependent helix–coil transition similar to collagen (Berg and Prockop, 1973a). However, the T_m for the helix–coil transition of the modified protocollagen was about 15° lower than that of collagen, suggesting that the presence of hydroxylated proline has a marked effect on the thermal stability of collagen.

Materials and Methods

Materials. White leghorn chick embryos were obtained from a local hatchery and they were incubated in a moist atmosphere at 37° until used. [^{14}C]Proline (213 Ci/mol) was purchased from the New England Nuclear Corp. Purified bacterial collagenase was purchased from either Sigma Chemical Co. (St. Louis, Mo.) or the Worthington Biochemical Co. (Freehold, N. J.). Trypsin solution, 2.5% in physiological saline, Eagle's minimum essential medium with glutamine for monolayer cultures, and fetal calf serum were purchased from Gibco Corp. (Grand Island, N. Y.). α-Chymotrypsin (46 units/mg) was purchased from the Worthington Biochemical Corp. and pepsin, twice recrystallized, was purchased from the Sigma Chemical Co.

Isolation of Cells. Tendons from 60 or 120 17-day-old chick embryos were removed by dissection and the matrix was

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digested by incubation with collagenase and trypsin under controlled conditions (Dehm and Prockop, 1971, 1972). The digestion was carried out for 35–45 min, or until the tissue was largely dispersed but a few intact pieces of tendon were still visible. The liberated cells were filtered through lens paper and collected by centrifugation at 600*g* for 6 min. The cells were washed three times with modified Krebs medium (Dehm and Prockop, 1971) containing 10% fetal calf serum.

To prepare [^{14}C]protocollagen, either about 0.8×10^9 cells from 60 embryos or about 1.5×10^9 cells from 120 embryos were incubated in a concentration of 7.5×10^6 cells/ml of modified Krebs medium containing 10% fetal calf serum in a siliconized erlenmeyer flask (Jimenez *et al.*, 1973). The cells were pre-incubated for 35 min with 0.2 mM α,α'-dipyridyl (Eastman Chemical Co.) with shaking at 37° and then either 10 or 60 μCi of [^{14}C]proline was added prior to continuing the incubation for an additional 4 hr. The samples were centrifuged at 1200*g* for 12 min at room temperature in order to separate the cells from the medium. The pellet containing the cells was cooled to 4° for 30 min, frozen at minus 20°, and then immediately homogenized in 20 ml of ice-cold 0.1 N acetic acid with a Teflon and glass homogenizer. The homogenate was dialyzed against 0.1 N acetic acid at 4° for 12 hr and centrifuged at 20,000*g* for 30 min. The supernatant was dialyzed against 0.4 M NaCl and 0.1 M Tris-HCl buffer, pH 7.9 at 4°, and the protein was precipitated with 176 mg/ml of ammonium sulfate (Baker Chemical Co.). The [^{14}C]protein in the cells, in the medium and in various fractions was assayed by dialyzing the samples extensively, hydrolyzing the retenates, and then measuring the ^{14}C in the hydrolysates with a liquid scintillation counter (see below).

To prepare [^{14}C]protocollagen-C,[1] the ammonium sulfate precipitate was suspended in 10 ml of the NaCl–Tris buffer (see above) at 4°, and then 300 μg/ml of α-chymotrypsin was added. The digestion was carried out by dialyzing the sample against the NaCl–Tris buffer at 4° for 15 hr and at 15° for 6 hr. To purify the [^{14}C]protocollagen-C from α-chymotrypsin and from peptides released by the digestion, the sample was precipitated with 176 mg/ml of ammonium sulfate and the pellet was dialyzed against 0.1 N acetic acid at 4°. The [^{14}C]-protocollagen-C was stored in 0.1 N acetic acid at 4°.

To prepare [^{14}C]protocollagen-P, the cells were extracted with cold acetic acid and the extract was precipitated with ammonium sulfate at neutral pH as described above. The ammonium sulfate precipitate was suspended in 10 ml of 0.1 N acetic acid and either 100 or 300 μg per ml of pepsin was added. The digestion was carried out by dialyzing against 0.1 N acetic acid at 4° for 15 hr and at 15° for 6 hr. The sample was then dialyzed against the NaCl–Tris buffer and precipitated with 176 mg/ml of ammonium sulfate. The precipitate was again dialyzed against the NaCl–Tris buffer and stored at 4°.

To prepare [^{14}C]procollagen-C and [^{14}C]procollagen-P, the procedures were the same as those used for the preparation of the [^{14}C]protocollagen-C and [^{14}C]protocollagen-P except that (a) the cells were incubated in modified Krebs medium without fetal calf serum and without any addition of α,α'-dipyridyl and (b) the medium was used as the source of the [^{14}C]procollagen. The medium was dialyzed against the NaCl–Tris buffer at 4° and precipitated with ammonium sulfate. The precipitate was then either suspended in 0.1 N acetic acid for pepsin digestion or in the NaCl–Tris buffer for α-chymotrypsin digestion. The conditions for the digestion were the same as described above except that the concentration of α-chymotrypsin was 100 μg/ml.

Hydroxylation with Prolyl-Hydroxylase. Prolyl-hydroxylase was prepared from 12-day-old chick embryos with the affinity column procedure recently described (Berg and Prockop, 1973b). Since there was a small decrease in activity of enzyme with time, especially after thawing and refreezing, it was imperative to assay the activity under standard conditions (Berg and Prockop, 1973b) in each experiment. The preparations at the time of use had specific activities ranging from 1500 to 2800 units per mg.

The enzymic reaction was carried out with 0.4–20 μg of [^{14}C]protocollagen, [^{14}C]protocollagen-C, or [^{14}C]protocollagen-P in a final volume of 4 ml with 5–160 units of enzyme and the concentrations of cofactors and cosubstrates as described previously (Berg and Prockop, 1973b). In some of the experiments involving hydroxylation of [^{14}C]protocollagen-P at different temperatures the salt concentration was increased to 0.4 M NaCl and 0.1 M Tris-HCl pH 7.9 at 4° to correspond to the conditions used for storing the [^{14}C]-protocollagen-P and carrying out the ultracentrifugation (see below). The results were indistinguishable from results obtained using the standard conditions of no added NaCl and 0.05 M Tris-HCl. The time of the reaction was varied from 10 to 60 min, depending on whether the experiments were designed to measure initial velocity or completeness of hydroxylation. The temperature of the reaction was varied from 15 to 37°. After reaction, the samples were immediately hydrolyzed in 6 N HCl and [^{14}C]hydroxyproline was assayed with a specific radiochemical procedure (Juva and Prockop, 1966).

Gel Filtration and Polyacrylalmide Gel Electrophoresis. For gel filtration in sodium dodecyl sulfate (Sigma Chemical Co.) 0.1 ml of [^{14}C]protein, containing 50,000–150,000 cpm, was mixed with concentrated stock solutions so as to give a solution of 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7.4) in a final volume of 2 ml. The samples were heated at 100° for 5 min and 2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, Wis.) was added to a final concentration of 1% for a further incubation at 37° for 4 hr. The samples were then directly applied to an sodium dodecyl sulfate–agarose column (Bio-Gel, A-5m, 200–400 mesh, Bio-Rad) which was 1.9×80 cm (Jimenez *et al.*, 1971). Fractions of 2.0 ml were collected and aliquots of 0.3 ml were mixed with 20 ml of a scintillation fluid previously described (Prockop and Ebert, 1963) and counted in a Beckman LS-133 liquid scintillation counter having an efficiency of about 80%. The recoveries of [^{14}C]protein eluted from the sodium dodecyl sulfate–agarose column were 89–95%.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out according to the method previously described (Weber and Osborn, 1969) with 5% polyacrylamide gels except that the stock solution was prepared with 11.1 g of acrylamide and 0.6 g of methylenebisacrylamide in 100 ml of distilled water. The electrophoresis was continued until the tracking dye reached the anodal end of the gels and the

[1] Abbreviations used are: 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 Methods); protocollagen-P, modified protocollagen prepared with the same procedures as protocollagen-C except that the limited digestion was carried out with pepsin instead of α-chymotrypsin; procollagen-C, modified procollagen prepared from the medium of matrix-free tendon cells and subjected to limited digestion with α-chymotrypsin; procollagen-P, modified procollagen prepared with the same procedures as procollagen-C except that the limited digestion was carried out with pepsin.

gels were then removed from the tubes and stained with Coomassie Brilliant Blue (R-250 Colab Laboratories, Chicago, Heights, Ill.) (Weber and Osborn, 1969).

For amino acid analysis, samples were hydrolyzed in 6 N HCl under N_2 for 15 hr at 120° and the analyses were carried out on a JOEL Model JLC-6AH automatic amino acid analyzer with a JOEL Model DK integrator. A single-column four-buffer system was employed and gave sufficient resolution to allow automated integration of all the amino acids found in collagen. The buffers were: 0.0668 M sodium citrate (0.2 N sodium citrate), pH 3.27; 0.0668 M sodium citrate, pH 4.25; 0.117 M sodium citrate, pH 5.28; and 0.468 M sodium citrate, pH 5.95. The column temperature was 49°. Prior to use, the buffers were passed through a cation-exchange resin (AG-50W-X12, 50–100 mesh, H form, Bio-Rad) to remove ammonia.

The specific activities of [^{14}C]proline in [^{14}C]protocollagen-C, [^{14}C]protocollagen-P, and [^{14}C]procollagen-C were measured by placing hydrolysates on the amino acid analyzer and splitting the stream of the column effluent for simultaneous ^{14}C assay of the proline peak with the scintillation system described above.

Molecular Weight Determination. Sedimentation equilibrium experiments were carried out according to the method of Yphantis (1964) except that double-sector synthetic boundary cells were employed. A Beckman Model E analytical ultracentrifuge equipped with Rayleigh interference optics was used. The sample compartment was filled to a column height of 3 mm and reference solution obtained by dialysis was placed in the reference compartment so that it layered over the sample during the run. The initial protein concentration was approximately 0.02%. The run was carried out at 15° with a speed of 11,249 rpm and photographs were taken each day for 3 days to determine when equilibrium was attained. The total time was 72.5 hr. Measurement of fringe dsiplacement as a function of radial distance was made with a Nikon Model 6 microcomparator, and the weight average molecular weight was calculated from the slope of a plot of log f (fringe displacement) against r^2 (radius squared). A partial specific volume of 0.706 (Rice *et al.*, 1964) was used for the calculation.

Results

Purification of Protocollagen-C and Protocollagen-P. To purify [^{14}C]protocollagen matrix-free tendon cells (1.4×10^9) were incubated for 4 hr with 10 μCi of [^{14}C]proline and 0.3 mM α,α'-dipyridyl. [^{14}C]Protocollagen was extracted from the cells and purified as described in the Materials and Methods. About 63% of the initial intracellular [^{14}C]protein was solubilized by extraction with cold acetic acid for 12 hr. After digestion with α-chymotrypsin, 29% of the initial [^{14}C]protein was recovered as [^{14}C]protocollagen-C. The specific activity of the [^{14}C]protocollagen-C was 5960 cpm/μg and about 500 μg was obtained from 1.4×10^9 cells in a typical experiment. Similar yields were obtained when [^{14}C]-protocollagen-P was prepared.

[^{14}C]Procollagen-C and [^{14}C]procollagen-P were prepared from the protein secreted into the medium of matrix-free cells incubated under control conditions without α,α'-dipyridyl. In a typical experiment 0.7×10^9 cells were incubated for 6 hr with 10 μCi of [^{14}C]proline. About 86% of the total [^{14}C]protein was recovered in the medium and 47% of the total [^{14}C]-protein was recovered by ammonium sulfate precipitation of the medium proteins. After digestion with α-chymotrypsin and reprecipitation with ammonium sulfate, about 30% of the original [^{14}C]protein was recovered as [^{14}C]procollagen-C. The specific activity of the [^{14}C]procollagen-C was 11,200 cpm/μg and about 322 μg was obtained from 0.7×10^9 cells. Similar results were obtained when [^{14}C]procollagen-P was prepared.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated that essentially all the protein in the preparations of [^{14}C]protocollagen-C and [^{14}C]procollagen-C was recovered in two bands which had previously been shown to have about the same mobilities as the α_1 to α_2 chains of collagen (Berg and Prockop, 1973a). By observation of the density of the stained bands it appeared that the ratio of α_1 to α_2 was about 2. When the polyacrylamide gels were cut into 1.5-mm slices and the slices were assayed for ^{14}C, all of the [^{14}C]protein

TABLE I: Amino Acid Analysis of Tendon Procollagen-C and Protocollagen-C.[a]

Amino Acid	Neutral Salt Soluble Collagen (Residues/1000)	Pro-collagen-C (Residues/1000)	Proto-collagen-C (Residues/1000)
Hydroxylysine	10	17	<1
Lysine	28	18	39
Histidine	5	6	4
Arginine	48	48	45
Cysteic acid	<4[b]	<4[b]	<1
Hydroxyproline	90	99	<1
Aspartic acid	51	36	51
Methionine	5	4	5
Threonine	18	18	22
Serine	27	25	35
Glutamic acid	83	73	79
Proline	115	122	208
Glycine	323	326	313
Alanine	116	127	119
Valine	20	23	24
Isoleucine	14	11	14
Leucine	29	28	30
Tyrosine	3	<1	<1
Phenylalanine	14	15	13
Proline *plus* hydroxyproline	205	221	208
Lysine *plus* hydroxylysine	38	35	39

[a] Amino acid analysis was carried out as described in the Materials and Methods. The values shown are the means of duplicate analyses on the same sample. The samples were [^{14}C]protocollagen-C and [^{14}C]procollagen-C from matrix-free tendon cells purified as in Materials and Methods. Neutral salt soluble collagen was purified (Jackson and Cleary, 1967) from the skin of 20-day-old chick embryos made lathyritic on day 18 by applying 10 mg of β-aminopropionitrile (Nutritional Biochemical Corp.) in 0.2 ml of H_2O to the air sac of each embryo. [b] Maximal values for small peaks which could not be accurately measured. Polyacrylamide gel electrophoresis of the neutral salt soluble collagen revealed two faint bands with the same mobilities as the pro-α chains of procollagen from tendon cells.

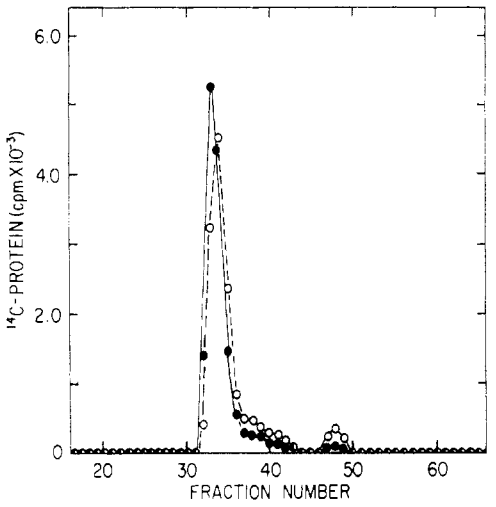

FIGURE 1: Digestion of [^{14}C]procollagen-C with α-chymotrypsin at 25° for 12 hr. [^{14}C]Procollagen-C, 95,000 cpm and 9 μg, was prepared as described in Materials and Methods, and incubated in 1 ml with or without 100 μg of α-chymotrypsin at 25° for 12 hr at pH 7.5. The samples were denatured and reduced with sodium dodecyl sulfate and mercaptoethanol, and they were chromatographed on a sodium dodecyl sulfate–agarose column (see Materials and Methods). The void volume was 48 ml (fraction 24) and the total volume was 136 ml (fraction 68). α chains of neutral salt soluble collagen from the skin of 20 day-old lathyritic chick embryos eluted as a peak in fraction 34. [^{14}C]Protein is shown as observed cpm in 0.3 ml of each 2.0 ml fraction. Control sample (●); sample incubated with α-chymotrypsin at 25° (○).

was recovered from the two bands corresponding to α_1 and α_2 chains of [^{14}C]protocollagen-C or [^{14}C]procollagen-C (Berg and Prockop, 1973a). Furthermore, gel filtration in sodium dodecyl sulfate–agarose indicated that essentially all of the ^{14}C in the enzyme modified protocollagen and procollagen eluted as a peak in the same position as the α chains of collagen (see below). Similar results were obtained with [^{14}C]protocollagen-P and [^{14}C]procollagen-P.

The amino acid composition of the [^{14}C]procollagen-C was similar to neutral salt soluble collagen from the skin of 20-day-old lathyritic chick embryos (Table 1). The procollagen-C had a higher ratio of hydroxylysine to lysine. Also,

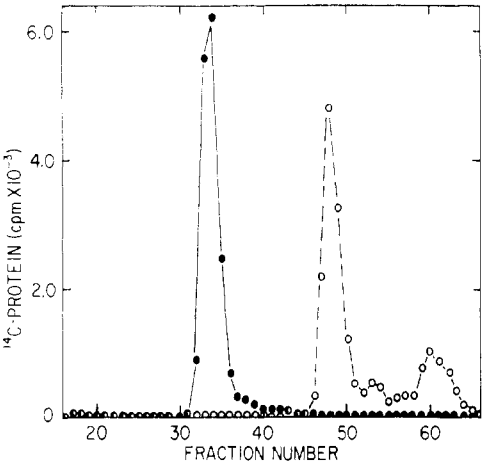

FIGURE 2: Digestion of [^{14}C]protocollagen-C with α-chymotrypsin at 25° for 12 hr. [^{14}C]Protocollagen-C, 130,000 cpm or 38 μg, was prepared as described in Materials and Methods. Other conditions were the same as in Figure 1. Control sample (●); sample incubated with α-chymotrypsin at 25° (○).

TABLE II: Hydroxylation of Native and Denatured Protocollagen-C at 15 and 37°.[a]

Pretreatment of [^{14}C]Protocollagen-C	100 × [^{14}C]Hydroxyproline/Total ^{14}C Hydroxylation at 15°	100 × [^{14}C]Hydroxyproline/Total ^{14}C Hydroxylation at 37°
Equilibrated at 15°	2.3	32.7
Heat denatured at 37° for 30 min	32.3	
Heat denatured at 100° for 5 min	28.2	29.2

[a] [^{14}C]Protocollagen-C, 19,000 cpm or about 10 nM, was hydroxylated with 15 units of prolyl-hydroxylase for 1 hr as described in Materials and Methods. The molar ratio of enzyme to [^{14}C]protocollagen-C was 0.60, so the extent of hydroxylation at 15 or 37° was about 70% maximal (see Figure 3).

it contained 15 residues/1000 less of aspartic acid, 10 residues less of glutamic acid, and 11 residues more of alanine. The amino acid composition of the [^{14}C]protocollagen-C was similar to [^{14}C]procollagen-C and neutral salt-soluble collagen from 20-day embryonic chick skin. The major difference was that the [^{14}C]protocollagen-C contained essentially no hydroxyproline or hydroxylysine and it was correspondingly rich in proline and lysine. The [^{14}C]protocollagen-C also contained 15 more residues/1000 of aspartic acid and 10 more residues of serine than the [^{14}C]procollagen-C. The reasons for the variations in amino acids other than hydroxylysine, lysine, proline, and hydroxyproline were not apparent.

The difference between the [^{14}C]protocollagen-C and [^{14}C]-procollagen-C was further demonstrated by measuring their resistance to proteolytic digestion as a function of temperature at acid and neutral pH. At 15° both the [^{14}C]protocollagen-C and the [^{14}C]procollagen-C were resistant to α-chymotrypsin. At 25° the [^{14}C]procollagen-C was largely resistant in that after incubation with 100 μg/ml of α-chymotrypysin for 12 hr, the polypeptide chains were still the same size as the α chains (Figure 1). In contrast, at 25° the [^{14}C]protocollagen-C was readily digested to small peptides (Figure 2). The latter observation is consistent with the recent demonstration that the T_m for [^{14}C]protocollagen-C in 0.1 N acetic acid is about 15° lower than that of [^{14}C]procollagen-C (Berg and Prockop, 1973a). Similarly, when [^{14}C]procollagen-P was incubated with 100 μg/ml of pepsin in 0.1 N acetic acid at 25° for 12 hr, there was little digestion of the α chain-size polypeptides. Under the same conditions [^{14}C]protocollagen-P was completely digested (not shown).

Hydroxylation of Native and Denatured Protocollagen. [^{14}C]Protocollagen-C which was prepared at 15° and stored at 4° in 0.1 N acetic acid was readily hydroxylated by prolyl-hydroxylase at 37° and at neutral pH (Table II). However, when the hydroxylation was carried out at 15°, [^{14}C]protocollagen-C was minimally hydroxylated[2] unless it was heat

[2] When the [^{14}C]protocollagen-P or the [^{14}C]protocollagen-C was hydroxylated at 15°, about 2% of the protein-bound [^{14}C]proline, or about 4 residues per α chain, were converted to hydroxyproline. This small degree of hydroxylation has not been examined and may be explained by small irregularities in the helical structure of the ends of the molecules produced by proteolytic digestion.

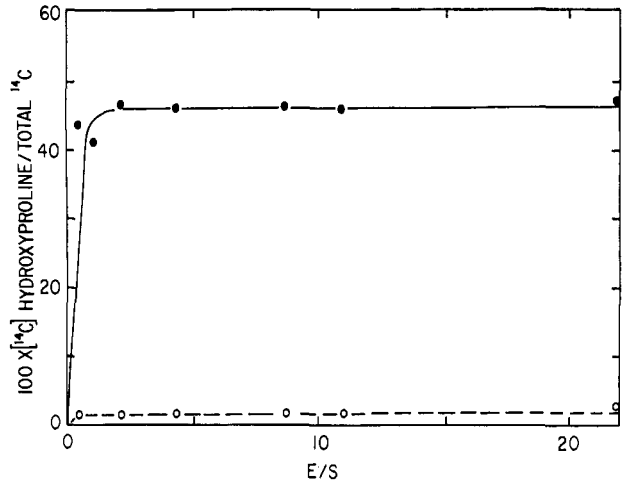

FIGURE 3: Hydroxylation of [^{14}C]protocollagen-P at 15° and at 37° as a function of enzyme concentration. The conditions for hydroxylation were as described in the Materials and Methods with a reaction time of 60 min and a volume of 4.0 ml. The substrate concentration was 2.89 nM polypeptide and the enzyme concentration was varied from 1.6 to 64 nM. The specific activity of the substrate was 19,700 cpm/μg (see Figure 5). [^{14}C]Protocollagen-P was either added directly to the reaction mixture at 15° and hydroxylated at 15° (○), or it was first denatured at 100° for 5 min and added to the reaction mixture at 37° for hydroxylation at 37° (●).

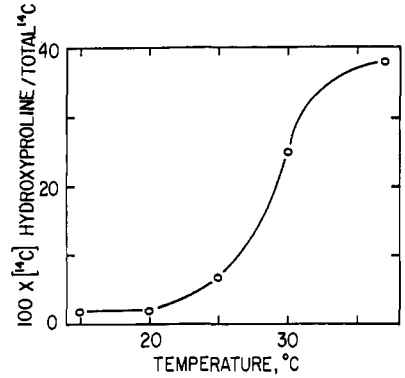

FIGURE 4: Hydroxylation of [^{14}C]protocollagen-P as a function of temperature. The substrate, prepared as described in Materials and Methods at 4°, was preincubated in the reaction mixture but without enzyme, iron, α-ketoglutarate, and ascorbic acid for 30 min at the indicated temperature. Enzyme and cofactors were added and hydroxylation was allowed to proceed for 60 min at the indicated temperature. The values shown are the mean of the values observed with two samples. In both samples the concentration of [^{14}C]protocollagen-P was 3.1 nM but in one sample the enzyme concentration was 2.2 nM (E:S of 0.72) and in the other sample the enzyme concentration was 9.0 nM (E:S of 2.90). The two values for each temperature agreed within 5%.

denatured by preincubation at either 37° or at 100° before the reaction with enzyme. Heat denaturation of [^{14}C]protocollagen-C increased the completeness of its hydroxylation at 15° by over 10-fold. After heat denaturation, the extent of the hydroxylation at 15° was the same as that observed at 37° with the same concentrations of enzyme and substrate. Similar results were obtained with [^{14}C]protocollagen-P (not shown), indicating that the substrate prepared by α-chymotrypsin treatment was similar to that prepared by pepsin treatment.

At 37° about 46% of [^{14}C]proline in [^{14}C]protocollagen-P was hydroxylated to [^{14}C]hydroxyproline in 1 hr when the reaction was carried out with a twofold molar excess of enzyme (Figure 3). As discussed elsewhere (Berg and Prockop, 1973b), this value indicates complete hydroxylation of [^{14}C]-proline in the "Y position" of the repeating -Gly-X-Y-triplets of the substrate. At 15° no more than 2% of the peptide-bound [^{14}C]proline[2] in the undenatured [^{14}C]protocollagen-C was hydroxylated even when the molar excess of enzyme was increased to 22 (Figure 3). Similar results were obtained with [^{14}C]protocollagen which was not purified and modified with α-chymotrypsin or pepsin (not shown), indicating that the resistance of the undenatured protein to hydroxylation at 15° was not influenced by the purification procedures.

In further experiments, the temperature of hydroxylation was varied from 15 to 37°. The results indicated a sharp change in the extent of hydroxylation of undenatured [^{14}C]-protocollagen-P between 25 and 30° (Figure 4). The sharp change in extent of hydroxylation with temperature was not explained by changes in the activity of the enzyme with temperature, since the same sharp transition was seen with a fourfold increase in the amount of enzyme. Also, with comparable concentrations of reactants heat-denatured [^{14}C]-protocollagen-C was readily hydroxylated at 15° (Table II).

The experiments with limited proteolysis described here and optical rotation experiments (Berg and Prockop, 1973a) indicated that the [^{14}C]protocollagen-C was helical at 15°. To exclude the possibility that the resistance of the [^{14}C]-protocollagen-P and [^{14}C]protocollagen-C to hydroxylation at 15° was explained by aggregation of the helical form, [^{14}C]protocollagen-P was examined by sedimentation equilibrium at 15° as described in Materials and Methods. The plot (not shown) of the log fringe displacement *vs.* radius squared was linear to the bottom of the cell, indicating that there was no gross aggregation of the protein at 15°, pH 7.6 and a concentration of 200 μg/ml. Furthermore, the calculated molecular weight was 283,000, a value which was similar to previous measurements of triple-helical collagen under similar conditions (Öbrink, 1972; Igarashi *et al.*, 1973).

Characterization of Random-Coil [^{14}C]Protocollagen as a Substrate for Prolyl-Hydroxylase. To measure the K_m for [^{14}C]protocollagen and the turnover number for prolyl-

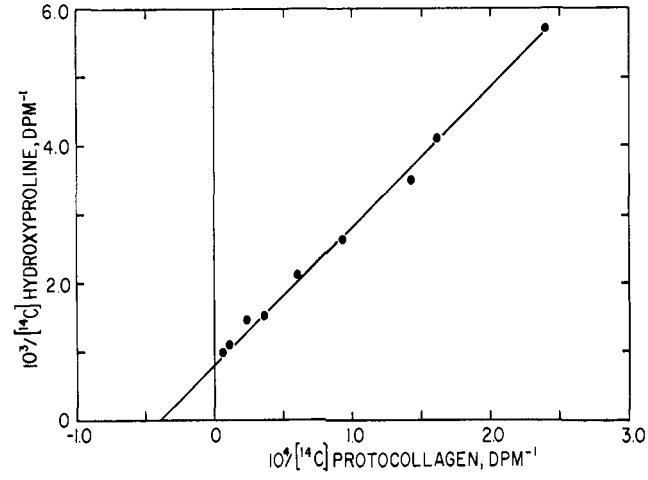

FIGURE 5: Double-reciprocal plot of initial velocities and substrate concentrations for the hydroxylation of [^{14}C]protocollagen with prolyl-hydroxylase. The conditions were as described in Materials and Methods. The volume was 4.0 ml, the time of incubation was 10 min, and the substrate was thermally denatured at 100° for 5 min immediately before addition to the reaction mixture. The specific activity of unpurified [^{14}C]protocollagen used as the substrate was 12,200 dpm/nmole of [^{14}C]proline or 19,700 cpm/μg of [^{14}C]protocollagen. The line is the calculated linear regression line for the values shown.

hydroxylase, the substrate employed was [^{14}C]protocollagen obtained by extracting the tendon cells with cold 0.1 N acetic acid and precipitating the protein with 30% ammonium sulfate from neutral solution (see Materials and Methods). The [^{14}C]protocollagen was not modified by proteolytic digestion but it was denatured by heating at 100° for 5 min immediately before hydroxylation with the enzyme at 37°. Double-reciprocal plots of initial velocities and substrate concentrations were linear (Figure 5) and the K_m was 2 nM calculated on the basis of a molecular weight of 100,000 per polypeptide chain. This value agrees reasonably well with previous estimates made with impure preparations of both enzyme and substrate (Kivirikko and Prockop, 1967), and it is consistent with earlier indications that the dissociation constant may be as low as 10^{-11} M (Juva and Prockop, 1969).

The specific activity of the [^{14}C]protocollagen used as a substrate for these experiments was determined by converting it to [^{14}C]protocollagen-P as described above and then measuring the specific activity of the [^{14}C]proline in a hydrolysate of the protein (see Materials and Methods). From the specific activities of the enzyme and [^{14}C]proline it was calculated that the turnover number for the enzyme was 4 sec^{-1} in terms of moles of hydroxyproline synthesized. This value agrees reasonably well with measurements of turnover numbers made with synthetic substrates for the enzyme (Berg *et al.*, 1973[3]).

Discussion

In the 1950's Gustavson originally suggested that hydroxyproline stabilizes the fibrillar structure of collagen (Gustavson, 1955). This suggestion was largely discounted by subsequent investigators (see Piez and Gross, 1960; Traub and Piez, 1971) but recently direct experimental evidence has been obtained for a role for hydroxyproline in stabilizing the intramolecular structure of collagen. In particular, the T_m of the helix–coil transition of [^{14}C]protocollagen-C was found to be 24°, a value which is about 15° lower than the T_m of [^{14}C]procollagen-C or of collagen (Berg and Prockop, 1973a). Further evidence for a role for hydroxyproline in stabilizing the collagen helix has been provided by studying synthetic peptides which are models for collagen (Sakakibara *et al.*, 1973). Both the synthetic polypeptides (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ underwent a temperature-dependent transition from triple-helical to randon coil forms, but the T_m for the peptide containing hydroxyproline was about 35° higher than the T_m for the peptide which did not contain hydroxyproline. From the results presented here it appears that not only does the hydroxylation of collagen affect the stability of its helical structure, but also the helical structure of collagen affects its ability to be hydroxylated by prolyl-hydroxylase.[4]

Extensive previous work (Drake *et al.*, 1966) on collagen demonstrated that the protein in its triple-helical conformation was resistant to proteolysis and this property has been recently used to study the conformation of procollagen (Layman *et al.*, 1971; Stark *et al.*, 1971). Protocollogen extracted with cold acetic acid from cells treated with α,α'-dipyridyl was shown to be resistant to proteolysis (Jimenez *et al.*, 1973) and this feature was used to purify protocollagen-P and protocollagen-C. Digestion of protocollagen preparations with either α-chymotrypsin or pepsin under conditions employed here removed contaminating proteins and the NH_2-terminal extensions on the pro-α chains but there was no hydrolysis of the collagen-like portion of the molecule. The protein obtained was pure by gel filtration and polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In addition, it had the expected amino acid composition in that it was similar to collagen with the major difference that it contained no hydroxylated proline or lysine.

Initially [^{14}C]protocollagen-P appeared to be the appropriate purified substrate to react with prolyl-hydroxylase, since any residual pepsin in the substrate would have been inactive at neutral pH. However, the procedures employed apparently removed the proteolytic enzymes efficiently and therefore similar results were obtained when [^{14}C]protocollagen-C, which was previously used to measure the thermal transition by optical rotation (Berg and Prockop, 1973), was used as a substrate (compare Figure 3 and Table II).

The data presented here demonstrate that hydroxylation of undenatured protocollagen by prolyl-hydroxylase is markedly temperature dependent. Undenatured [^{14}C]protocollagen-C or [^{14}C]protocollagen-P did not serve as a substrate below 25° even though heat denatured preparations were readily hydroxylated under the same conditions. In addition, there was a marked change in the ability of the [^{14}C]protocollagen-P or [^{14}C]protocollagen-C to serve as a substrate for prolyl-hydroxylase at temperatures close to the temperature of the thermal transition seen by optical rotation (Berg and Prockop, 1973a). The results indicated therefore that the structure of protocollagen is critical in determining its ability to be hydroxylated.

Sufficient amounts of [^{14}C]protocollagen-P and [^{14}C]protocollagen-C were purified to make critical comparisons of their conformation with that of collagen. At 15° the [^{14}C]-protocollagen preparations showed the same resistance to proteolysis under acid conditions by pepsin (Jimenez *et al.*, 1973) or at neutrality by α-chymotrypsin (above) as collagen. Below 20° protocollagen-C had the same specific optical rotation as collagen (Berg and Prockop, 1973a). In addition, the resistance to proteolysis and the specific optical rotation of the enzyme-modified protocollagen showed the same temperature-dependent changes as collagen except that both changes occurred at lower temperatures. These observations together with the chemical similarities to collagen indicated that under the conditions where it resisted enzymic hydroxylation, the enzyme-modified [^{14}C]protocollagen had a helical conformation similar if not identical to the triple-helical conformation of collagen.

Since collagen itself forms aggregates under some conditions (see Öbrink, 1972; Traub and Piez, 1971) it was necessary to consider the possibility that at 15–20° the enzyme-modified [^{14}C]protocollagen was not only helical but also present as aggregates, and that it was the formation of the aggregates which made the substrate inaccessible to enzyme. There was however no evidence of aggregation of protocollagen-P by sedimentation equilibrium at 15°, pH 7.6, and at a concentration which was 40-fold greater than that used in the enzymic hydroxylation. Accordingly, the 95% decrease in the ability of the [^{14}C]protocollagen preparations to be hydroxylated at 15° cannot be explained by gross aggregation of the protein. We conclude therefore that the helical conformation of the enzyme-modified protocollagen was the critical feature which prevented its hydroxylation by prolyl-hydroxylase.

[3] R. A. Berg, Y. Kishida, S. Sakakibara, and D. J. Prockop, in preparation.

[4] Recent observations with lysyl-hydroxylase indicate that the lysyl residues in collagen can be further hydroxylated but the hydroxylation occurs only if the collagen is first heat-denatured (K. I. Kivirikko, L. Ryhanen, H. Anttinen, P. Bornstein, and D. J. Prockop, in preparation).

Previous observations (Nordwig and Pfab, 1968; Hutton *et al.*, 1967; Kivirikko *et al.*, 1968) suggesting triple-helical protocollagen can be hydroxylated must be reinterpreted because the enzymic hydroxylations were carried out at 30 and 37°, temperatures now known to be above the T_m of the triple-helical molecule. Studies with synthetic peptides resembling collagen suggested that these peptides could be hydroxylated in the triple-helical conformation, but it was difficult to ensure that the peptides were entirely triple helical and soluble during the enzymic reaction (Kivirikko *et al.*, 1972; Kikuchi *et al.*, 1969). Experiments on the further hydroxylation of heat denatured extracellular collagen with excess prolyl-hydroxylase (Rhoads *et al.*, 1971) were consistent with the observations here.

The results presented here have several consequences concerning current concepts about hydroxyproline formation during collagen biosynthesis. Since helical protocollagen is not hydroxylated by the prolyl-hydroxylase, it appears that the hydroxylation of peptide-bound proline during collagen biosynthesis must occur on random-coil chains.[5] Since intracellular [^{14}C]protocollagen can be hydroxylated up to 2 hr after it is synthesized (see Grant and Prockop, 1971), it appears that it must remain in a random-coil conformation until hydroxylation. Conversely, the data suggest that if the polypeptides become helical, hydroxylation of prolyl residues ceases. On this basis the formation of the triple helix may limit the extent to which prolyl residues in the -Gly-X-Pro- triplets of collagen are hydroxylated and therefore the amount of hydroxyproline introduced into the molecule before it is secreted. This observation may account for the variation in the hydroxylation of specific proline residues in collagen (for review of collagen sequences, see Traub and Piez, 1971).

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[5] A recent report (Jimenez *et al.*, 1973) concluding that protocollagen accumulating in matrix-free cells is in the triple-helical form is probably in error since the helical conformation was measured by susceptibility to proteolytic digestion by pepsin after the intracellular proteins were extracted in cold 0.1 N acetic acid for long times. The observations presented here and elsewhere (Uitto and Prockop, 1973) suggest that protocollagen containing the H_2N-terminal extensions becomes helical when cooled to 15° or less.