Hydroxylation of Prolyl Residues in Type II Procollagen in Vitro and in Cellulo. Lack of Preferential Hydroxylation of Specific Regions of the Protein†

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ABSTRACT: [^{14}C]Proline-labeled protocollagen, the unhydroxylated form of procollagen, was isolated from cartilage cells incubated with α,α'-dipyridyl. For examination of the initial steps in the hydroxylation of the protein, it was incubated in vitro with prolyl hydroxylase so that an average of 1.3–2.7 prolyl residues per chain was hydroxylated. The partially hydroxylated α chains were cleaved with cyanogen bromide, and the fragments were separated by polyacrylamide gel electrophoresis or column chromatography. The cyanogen bromide fragments were hydroxylated to the same degree. The results indicated, therefore, that in the initial hydroxylation of α chains in vitro, there was no preferential hydroxylation of any specific regions of the protein. In a second series of experiments, cartilage cells were incubated with [^{14}C]proline and α,α'-dipyridyl so that prolyl hydroxylase in the cells was extensively, but not completely, inhibited. Partially hydroxylated α chains were isolated, and cyanogen bromide fragments of the α chains from the cells were assayed for hydroxy[^{14}C]proline. The α chains contained an average of two residues of hydroxyproline per chain, and the cyanogen bromide fragments were hydroxylated to about the same degree. The results indicated, therefore, that when prolyl hydroxylase activity in cells is low relative to the rate at which pro α chains are synthesized, hydroxylation of prolyl residues occurs as it does in vitro, and there is no preferential hydroxylation of a specific region of the protein.

The hydroxyproline in procollagen and collagen is synthesized by the hydroxylation of peptidyl prolyl residues by prolyl hydroxylase [for reviews, see Cardinale & Udenfriend (1974) and Prockop et al. (1976, 1979)]. The enzyme (EC 1.14.11.2) is found in the cisternae of the rough endoplasmic reticulum, and in unperturbed cells, hydroxylation of prolyl residues begins as nascent pro α chains of procollagen are still being assembled (Peterkofsky & Udenfriend, 1963; Lazarides et al., 1971; Diegelmann et al., 1973; Peterkofsky & Assad, 1976; Uitto & Prockop, 1974a,b; Brownell & Veis, 1975). In some experimental conditions, however, hydroxylation is delayed and can apparently occur after complete assembly of pro α chains and the synthesis of interchain disulfide bonds in the carboxy-terminal propeptides [see Prockop et al. (1976, 1979)].

In the present paper, we have examined the initial hydroxylation of α chains in vitro to determine whether any region of the protein is preferentially hydroxylated. We have also explored the question of whether there is preferential hydroxylation of any region of the protein in cellulo when prolyl hydroxylase is extensively inhibited with α,α'-dipyridyl.

Experimental Procedures

Materials. Unless otherwise indicated, materials were obtained from sources previously identified (Kao et al., 1979).

Preparation of [^{14}C]Proline-Labeled Protocollagen-P.[1] Type II protocollagen-P was prepared from cartilage cells with minor modifications of the procedure previously used to prepare type I protocollagen-P tendon cells (Berg & Prockop, 1973a,b). Sternal cartilages from 270 17-day-old chick embryos were digested with bacterial collagenase and trypsin to obtain cartilage cells free of extracellular matrix (Dehm & Prockop, 1971). The cells, about 2.4×10^9, were incubated in 240 mL of modified Krebs medium containing 20% fetal calf serum and 0.5 mM α,α'-dipyridyl. After preincubation in the medium for 30 min, 100 μCi of [^{14}C]proline was added, and the incubation was continued for 3 h. Cycloheximide, 100 μg/mL, was then added to inhibit further protein synthesis. The cell suspension was cooled at 4 °C for 15 min, and the cells were removed by centrifugation at 1300g for 6 min. The cell pellet was homogenized at 4 °C in 20 mL of 0.5 M acetic acid with a Teflon and glass homogenizer. The homogenate was dialyzed overnight against 0.5 N acetic acid at 4 °C and centrifuged at 12000g for 30 min. Pepsin, 100 μg/mL, was added to the supernatant, and the sample was dialyzed against 0.5 N acetic acid at 4 °C for 15 h. After the pepsin digestion, the sample was dialyzed against 0.1 M Tris-HCl buffer, pH 7.5 at 4 °C, containing 0.4 M NaCl. Protein in the sample was precipitated by adding 176 mg/mL ammonium sulfate (Baker Chemical Co.) and centrifuging at 12000g for 30 min. The precipitate was dissolved in 11 mL of 0.1 M Tris-HCl buffer containing 0.4 M NaCl. The protein was reprecipitated by adding NaCl to a final concentration of 20 g/100 mL and stirring the sample at 4 °C for 15 h. The sample was centrifuged at 12000g for 30 min, and the pellet was dissolved in 2 mL of 0.1 M Tris-HCl buffer containing 0.4 M NaCl, before being frozen for storage.

Hydroxylation of [^{14}C]Protocollagen-P with Purified Prolyl Hydroxylase. Prolyl hydroxylase was a generous gift from Dr. Richard A. Berg. The enzyme was purified from chick embryos by affinity chromatography and was homogeneous by the criteria presented previously (Berg & Prockop, 1973a,b). In initial experiments to determine the appropriate conditions for initial hydroxylation of the substrate, the enzymic reaction was carried out in a volume of 1 mL containing about 9×10^4 cpm of [^{14}C]protocollagen-P and 0.14–1.2 units of prolyl hydroxylase. In subsequent experiments, the hydroxylation was carried out in a final volume of 4.0 mL con-

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[1] Abbreviations: protocollagen-P, pepsin-treated protocollagen; CNBr, cyanogen bromide; CB1–CB14, cyanogen bromide peptides from α1(II) chains; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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taining about 2.4×10^6 cpm of [^{14}C]protocollagen-P and 13 units of prolyl hydroxylase. The concentration of cofactors and cosubstrates was as described previously (Berg & Prockop, 1973a,b) except that catalase and bovine serum albumin were omitted from the reaction mixture. The enzymic reaction was carried out at 37 °C for 10 min, and the incubation was stopped by adding 0.1 volume of 10 mM α,α'-dipyridyl and cooling the samples to 4 °C.

For assay of hydroxy[^{14}C]proline content of the hydroxylated [^{14}C]protocollagen-P, the samples were hydrolyzed in 6 N HCl at 116 °C for 18 h, and the hydrolysates were assayed both with a specific radiochemical procedure (Juva & Prockop, 1966) and by ion-exchange chromatography (see below). For preparation of the CNBr peptides from the hydroxylated [^{14}C]protocollagen-P, the reaction mixture was dialyzed against 0.1 M Tris-HCl buffer containing 0.4 M NaCl and then against distilled water. The samples were lyophilized and stored at −20 °C until used for CNBr digestion (see below).

Intracellular Hydroxylation of [^{14}C]Protocollagen-P. In preliminary experiments to test the effect of varying concentration of α,α'-dipyridyl, about 8×10^8 cartilage cells were incubated in 100 mL of modified Krebs medium containing 20% fetal calf serum and varying concentrations of α,α'-dipyridyl. After preincubation for 17 min, 50 μCi of [^{14}C]proline was added to each flask and the incubation continued for 2 h. Cycloheximide, about 100 μg/mL, was then added, and the cells were separated from the medium by centrifugation at 1300*g* for 6 min. The cell pellet was cooled at 4 °C for 15 min, homogenized in 0.5 N acetic acid, and digested with pepsin as described above for the preparation of [^{14}C]protocollagen-P. The pepsin-resistant protein was purified by ammonium sulfate precipitation and sodium chloride precipitation, and then it was hydrolyzed in 6 N HCl for assay of hydroxy[^{14}C]proline (see below).

In subsequent experiments in which CNBr peptides were prepared, about 2.2×10^9 cells were incubated in above 300 mL of modified Krebs solution containing 20% fetal calf serum, 100 μCi of [^{14}C]proline, and 0.15 mM α,α'-dipyridyl for 2 h. The cells were separated, and pepsin-resistant protein was purified by the procedures described above for the purification of [^{14}C]protocollagen-P. After pepsin digestion and precipitation with ammonium sulfate and NaCl, the samples were dissolved in 0.1 M Tris-HCl buffer containing 0.4 M NaCl and then dialyzed against distilled water. The samples were lyophilized and stored at −20 °C until used for CNBr digestion.

Preparation of CNBr Peptides. Lyophilized samples of hydroxylated [^{14}C]protocollagen-P which were prepared as described above were mixed with 30 mg of type II collagen. The type II collagen was a gift from Dr. Jouni Uitto and was prepared from sternal cartilages from 17-day-old chick embryos by the procedure of Miller (1972). Each sample was dissolved in 2 mL of 70% formic acid and flushed with nitrogen. About 10 mg of CNBr was added, and digestion was carried out at 30 °C for 4 h. The sample was centrifuged at 12000*g* for 15 min, and the supernatant was diluted with 9 mL of distilled water. Excess CNBr was removed with a water aspirator, the sample was lyophilized, and the residue was stored at −20 °C.

Separation of CNBr Peptides by Gel Electrophoresis. Each sample of CNBr peptides was dissolved in 1.5 mL of 10% $NaDodSO_4$, and the sample was heated at 100 °C for 3 min followed by heating at 37 °C for 2 h. For visualization of the CNBr peptides from the carrier collagen, the gels were stained with Coomassie Brilliant Blue and destained (Hoffman et al., 1976). For location of the major bands of ^{14}C-labeled protein in the gels, the gel was cut into 1-mm slices with a Mickle gel slicer. Each was suspended in 0.4 mL of a solution of Protosol (New England Nuclear Corp.)–H_2O–toluene (9:1:10), and the sample was incubated at 37 °C for 15 h. The sample was cooled to 4 °C overnight, and 15 mL of a phosphor solution containing methyl Cellosolve and toluene was added. The sample was placed in the dark for about 5 h and ^{14}C assayed with a liquid scintillation counter. The remaining gels were cut into 3-mm slices, and on the basis of the results obtained by staining and counting the first gel, appropriate slices were pooled so as to recover the major peaks of ^{14}C-labeled protein. The pooled gels were hydrolyzed in 6 N HCl at 116 °C for 18 h. The total sample was evaporated in an evaporating dish on a steam bath, and the residue was dispersed in 1 mL of distilled water. The solution was removed with a Pasteur pipet, and gel slices were washed 3 more times with 1 mL of distilled water. The combined extracts were then assayed for total ^{14}C content and for hydroxy[^{14}C]proline as described below.

Chromatography of CNBr Peptides on CM-cellulose. Chromatography of CNBr peptides was carried out on CM-cellulose (CM-52; Whatman) according to the procedure of Miller et al. (1973). In initial experiments, a column 0.9 cm in diameter and 8 cm high was employed. The column was eluted at room temperature with a linear gradient prepared with 200 mL of 0.02 M sodium citrate buffer, pH 3.6, containing 0.01 M NaCl, and 200 mL of the same buffer, containing 0.16 M NaCl. The flow rate of the column was about 50 mL/h, and fractions of 5 mL were collected. In subsequent experiments, a 0.9×13 cm column was used, and the gradient was prepared with 300 mL of each of the two buffers. The peaks of peptides eluted from the column were desalted on a 1.8×25 cm column of polyacrylamide (Bio-Gel P-2; Bio-Rad) equilibrated and eluted with 0.2 N acetic acid. The samples were then lyophilized.

Assays of Hydroxy[^{14}C]proline and [^{14}C]Proline. For assay of hydroxy[^{14}C]proline, all the samples were hydrolyzed in 6 N HCl at 116 °C for 15 h, and the hydrolysates were evaporated in an evaporating dish on a steam bath. The samples were dissolved in distilled water, and one aliquot of each sample was assayed for hydroxy[^{14}C]proline with a specific radiochemical assay which involves oxidation of hydroxyproline to pyrrole (Juva & Prockop, 1966). A separate aliquot of the same sample was assayed by ion-exchange chromatography. Chromatography was carried out on a 0.9×45 cm column of cation-exchange resin (custom spherical resin type AA-15; Beckman) which was equilibrated and eluted with 0.2 M sodium citrate buffer, pH 3.05.

Identification of CNBr Peptides by Gel Electrophoresis. For characterization of the CNBr peptides eluted from the CM-cellulose column, electrophoresis was carried out on slab gels according to the procedure of King & Laemmli (1971). The stacking gel was prepared with 4.5% polyacrylamide and the separating gel with 10% polyacrylamide. Electrophoresis was carried out at about 50 mA for 5 h at 25 °C. Samples of CNBr peptides were lyophilized and dissolved in 0.5 mL of 0.125 M Tris-HCl buffer, pH 6.8, which contained 2% $NaDodSO_4$, 10% glycerol, and 0.001% bromophenol blue. The samples were heated at 100 °C for 3 min, and 100–150 μL was applied to the gels. Fluorographs were prepared, and they were exposed to RP-Royal "XOMAT" X-ray film at −70% for about 48 h (Hoffmann et al., 1976). The autoradiograph was scanned by attaching 6-mm-wide strips of X-ray film to the wall of a 10-cm-long cuvette and scanning the strips with

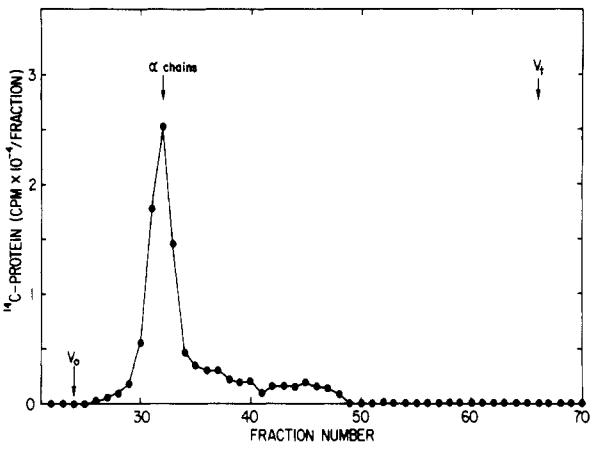

FIGURE 1: Gel filtration in $NaDodSO_4$ of α chains of protocollagen-P. V_0 and V_t indicate void volume and total volume, respectively.

a Gilford ultraviolet spectrophotometer and a linear transport accessory designed for scanning cylindrical gels.

Results

Initial Hydroxylation of [^{14}C]Proline-Labeled Protocollagen by Purified Prolyl Hydroxylase. For experiments with purified prolyl hydroxylase, the substrate employed was pepsin-modified [^{14}C]proline-labeled protocollagen. About 2.4 $\times$ 10^9 cartilage cells from 17-day-old chick embryos were incubated with 100 μCi of [^{14}C]proline for 3 h, and (1.8–2.0) $\times$ 10^7 cpm of ^{14}C-labeled protein was recovered in cell homogenates. After digestion of the homogenates with pepsin, (4.2–5.6) $\times$ 10^6 cpm of protocollagen-P was recovered. Gel filtration in $NaDodSO_4$ (Figure 1) demonstrated that most of the protein was recovered as intact α chains. Assays on acid hydrolysates of the protein (see below) demonstrated that less than 0.2% of the total ^{14}C was hydroxyl[^{14}C]proline. Over 95% of the remaining ^{14}C was recovered as [^{14}C]proline.

A series of trial experiments were carried out to establish conditions for obtaining partial hydroxylation of the protocollagen-P by prolyl hydroxylase. About 0.8% of the prolyl residues were hydroxylated with 0.15 unit of enzyme under the conditions employed here. About 1.4% were hydroxylated with 0.4 unit of enzyme and 2.2% with 1.2 units. Although the reaction was not linear over a broad range, it was possible to adjust enzyme and substrate so that the desired degree of hydroxylation was readily obtained.

For examination of the initial hydroxylation, the reaction was carried out so that the substrate was hydroxylated to a final level of either 0.6, 1.1, or 1.3%. Calculated on the basis of a total imino acid content of 210 residues (Miller, 1972), the products of the reaction contained on the average 1.3, 2.3, and 2.7 residues of hydroxyproline per α1(II) chain.

The α chains were digested with CNBr and the CNBr peptides were isolated either by polyacrylamide gel electrophoresis in $NaDodSO_4$ or by chromatography on CM-cellulose.

Separation of the CNBr peptides by polyacrylamide gel electrophoresis on cylindrical gels allowed isolation of CB10 and CB11 (Figure 2), large peptides which originate from the central region of the α chain (see below). Peptides CB7, CB8, CB9, and CB12 were recovered as unresolved peaks.

Separation of the CNBr peptides on CM-cellulose permitted a more complete fractionation of the peptides (Figure 3). The identity of each of these peaks was established by comparison with results published by Miller et al. (1973) and Byers et al. (1974) and by examination of the eluted peaks by electrophoresis (see below). The first peak contained CB4, CB6, and

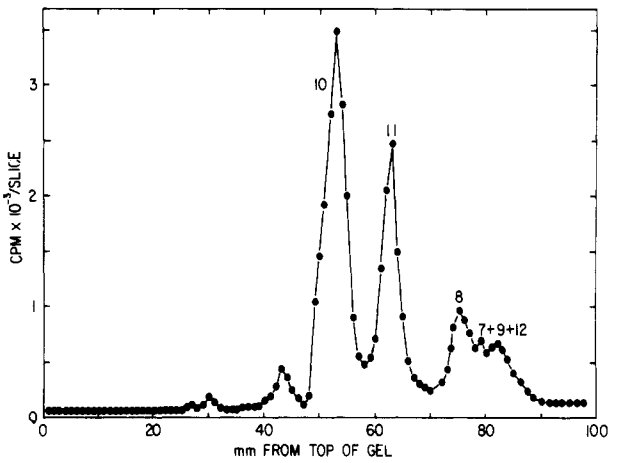

FIGURE 2: Separation of CNBr peptides of partially hydroxylated protocollagen-P by gel electrophoresis.

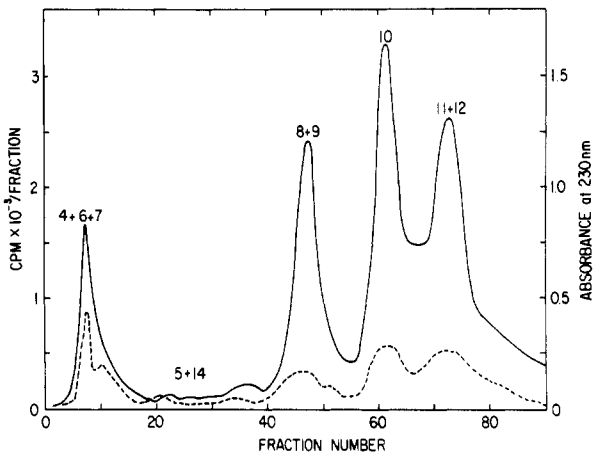

FIGURE 3: Separation of CNBr peptides of partially hydroxylated protocollagen-P by chromatography on CM-cellulose. Symbols: cpm/fraction (—); absorbance at 230 nm (- - -).

CB7, small peptides from the two ends of the α chains. Since CB4 does not contain proline (Miller et al., 1973), it was ignored in estimates for degree of hydroxylation of CB6 (Table I). CB7 eluted in the trailing edge of the first peak. CB5 and CB14, small peptides from near the carboxy-terminal end of the α chain, were recovered in a poorly resolved region of the chromatograph. Because they contained a total of only 30 amino acid residues, no effort was made to purify these two peptides further, and they were not included in the analyses presented here. Relatively distinct peaks were obtained containing CB8 plus CB9, CB10, and CB11 plus CB12. The separation achieved was similar to that reported by Miller et al. (1973), except that the separation of CB7 from CB4 and CB6 was not as complete. Also, CB9 appeared in the same peak as CB8 and did not cochromatograph with CB10 (see below).

The degree of hydroxylation of prolyl residues in the isolated peptides was determined by hydrolyzing the peptides and assaying the hydrolysates by ion-exchange chromatography and by a specific radiochemical procedure. Chromatography of the hydrolysates on a cation-exchange column allowed adequate separation of hydroxy[^{14}C]proline and [^{14}C]proline (Figure 4). Although the degree of hydroxylation of the peptides was low, it was possible to take adequate amounts for the assays so that the observed values for ^{14}C in hydroxyproline in both assays were over 10 times the background levels. As indicated in Table I, values for degree of hydroxylation obtained by the radiochemical assay were about

Table I: Distribution of Hydroxy[^{14}C]proline in Protocollagen-P after Hydroxylation in Vitro[a]

separation techniques	peptides	degree of hydroxylation of prolyl residues[b] (%) chromatographic assay	radiochemical assay	corrected values[d]
$NaDodSO_4$-PAGE[e]	α chain	0.65		0.69
	CB10	0.59		0.65
	CB11	0.61		0.60
	CB7 + CB8 + CB9 + CB12	0.61		0.60
	α chain	1.07	1.10	1.14
	CB10	1.06	1.10	1.19
	CB11	1.12	1.05	1.07
	CB7 + CB8 + CB9 + CB12	1.16	1.12	1.13
CM-cellulose chromatography	α chain		1.30	1.38
	CB6[c]		1.45	1.74
	CB7		1.68	1.68
	CB8 + CB9		1.18	1.30
	CB10		1.56	1.72
	CB11 + CB12		1.33	1.24

[a] ^{14}C-Labeled protocollagen-P was hydroxylated with prolyl hydroxylase (Figure 2), the protein was digested with cyanogen bromide, and CB peptides were isolated either by gel electrophoresis (Figure 2) or CM-cellulose chromatography (Figure 3). For conditions, see text. [b] Degree of hydroxylation was calculated as 100([^{14}C]hydroxyproline content/[^{14}C]hydroxyproline plus [^{14}C]proline content). As indicated, [^{14}C]hydroxyproline content of peptides was assayed with both a specific radiochemical assay and ion-exchange chromatography (see Experimental Procedures). [c] Fractions of CB6 (Figure 3) also contained CB4, but since CB4 does not contain proline (Miller et al., 1973), it did not affect the value for the degree of hydroxylation of CB6. [d] Observed values for degree of hydroxylation were corrected for variations in hydroxylatable proline residues by multiplying observed values by 0.5 × the ratio of total imino acids to the hydroxyproline content of the peptide (Miller, 1971, 1972). The correction factors were 1.06 for the α1(II) chain, 1.10 for CB10, 0.98 for CB11, 0.99 for CB7 + CB8 + CB9 + CB12, 1.20 for CB6, 1.00 for CB7, 1.10 for CB8 + CB9, and 0.93 for CB11 + CB12. [e] PAGE, polyacrylamide gel electrophoresis.

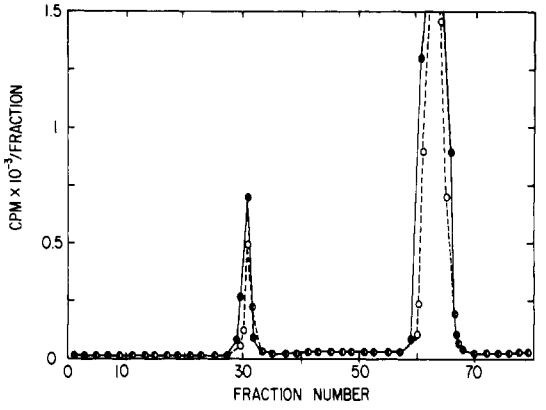

FIGURE 4: Assay of hydroxy[^{14}C]proline and [^{14}C]proline by ion-exchange chromatography. Data obtained with hydrolysates of two separate samples are shown.

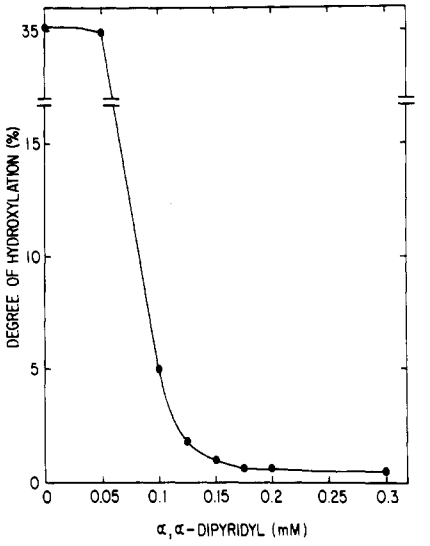

FIGURE 5: Degree of hydroxylation of intracellular protein as a function of the concentration of α,α′-dipyridyl in the incubation medium. Values are expressed as 100 (hydroxy[^{14}C]proline/total ^{14}C).

the same as values obtained by the chromatographic assay. Also as indicated in Table I, the degree of hydroxylation of the various CNBr peptides was about the same as the overall hydroxylation of the α chain. In the experiment in which the corrected degree of hydroxylation of the α chains of protocollagen-P was 0.69%, the degree of hydroxylation of three fractions of peptides separated by $NaDodSO_4$–polyacrylamide gel electrophoresis varied from 0.60 to 0.65%. In the second experiment, the corrected degree of hydroxylation of the α chains was 1.14%, and the degree of hydroxylation of three fractions of peptides was 1.07–1.19%. Similar results were obtained in a third experiment in which the CNBr peptides were isolated by chromatography on CM-cellulose instead of by electrophoresis. The corrected hydroxylation of the α chains was 1.38%, and the degree of hydroxylation of five fractions of CNBr peptides varied from 1.24 to 1.74%.

Intracellular Hydroxylation of [^{14}C]Procollagen. In initial experiments conditions were established for partially inhibiting prolyl hydroxylase in cartilage cells synthesizing type II procollagen. As indicated in Figure 5, the degree of hydroxylation of the intracellular protein was decreased by concentrations of α,α′-dipyridyl greater than 0.05 mM. With a concentration of about 0.15 mM α,α′-dipyridyl, the overall degree of hydroxylation of intracellular polypeptides was about 1%, and this concentration was employed for further experiments on the intracellular hydroxylation.

Cartilage cells were incubated with 0.15 mM α,α′-dipyridyl for 2 h, the cells were cooled to 4 °C, and the partially hydroxylated intracellular protocollagen-P was isolated by the procedures used previously to purify protocollagen-P. CNBr peptides were prepared and isolated by chromatography on CM-cellulose. Apparently because a larger column was used than in Figure 3, the separation of some of the peptides was more complete (Figure 6). The composition of each peak was established by polyacrylamide gel electrophoresis in $NaDodSO_4$ (Figure 7). Pooled fractions containing CB4 and CB6 migrated at the dye front (not shown) and were free of CB7.

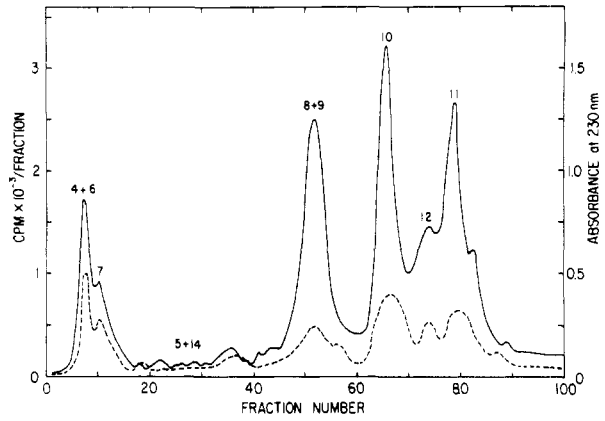

FIGURE 6: Chromatography on CM-cellulose of CNBr peptides of partially hydroxylated protocollagen-P from cells incubated with 0.15 M α,α'-dipyridyl. Symbols: cpm/fraction (—); absorbance at 230 nm (---).

Table II: Distribution of Hydroxy[^{14}C]proline in Protocollagen-P after Hydroxylation in Cells[a]

peptides	imino acid content[b] Pro	Hyp	degree of hydroxylation of prolyl residues: chromatographic assay	radiochemical assay	corrected values[e]
α chain	118	106	1.10	1.15	1.20
CB6[c]	7	5	0.90	1.10	1.20
CB7	8	8	1.24	1.10	1.17
CB8[d]	14	16	0.88		0.83
CB9	10	4	0.99		1.73
CB10	36	30	0.90	1.17	1.13
CB12	6	11	0.98	1.13	0.88
CB11	29	30	0.94	1.30	1.10

[a] Cells were incubated with [^{14}C]proline and 0.15 mM α,α'-dipyridyl as described in the text. Partially hydroxylated protocollagen-P was isolated from the cells. The protein was cleaved with CNBr and the CNBr peptides were isolated on CM-cellulose as indicated in Figure 6. [b] Values are expressed as residues of peptide and were taken from Miller (1971, 1972). [c] Fractions of CB6 (Figure 6) also contained CB4, but since CB4 contains no proline (Miller et al., 1973), it did not affect the value for the degree of hydroxylation of CB6. [d] Value for CB8 was calculated from the value for CB9 and the value 0.94 for the mixture of CB8 plus CB9 obtained with the chromatographic assay. Radiochemical assay of the mixture gave a value of 0.91. [e] Mean or individual values were corrected as described in Table I. The additional correction factors used here were 1.00 for CB7, 0.94 for CB8, 1.75 for CB9, and 0.77 for CB12.

Since CB4 does not contain proline or hydroxyproline, the sample was not purified further. CB7 migrated as a single band on electrophoresis (Figure 7B). CB8 and CB9 were recovered in a single peak, and therefore they were assayed both as a pooled fraction and after separation of CB9 from CB8 by gel filtration (Figure 7). The three peptides CB10, CB12, and CB11 migrated as single bands on electrophoresis.

Values for degree of prolyl hydroxylation were essentially the same when the peptides were assayed with either the chromatographic assay or the radiochemical assay (Table II). As indicated, the corrected degree of hydroxylation for the α chain was about 1.2%, and values for individual peptides ranged from 0.83 to 1.73%. Arrangement of the peptides in the established order for the α chain (Figure 8) indicated that the hydroxy[^{14}C]proline was uniformly distributed.

Discussion

During procollagen biosynthesis, prolyl residues in about

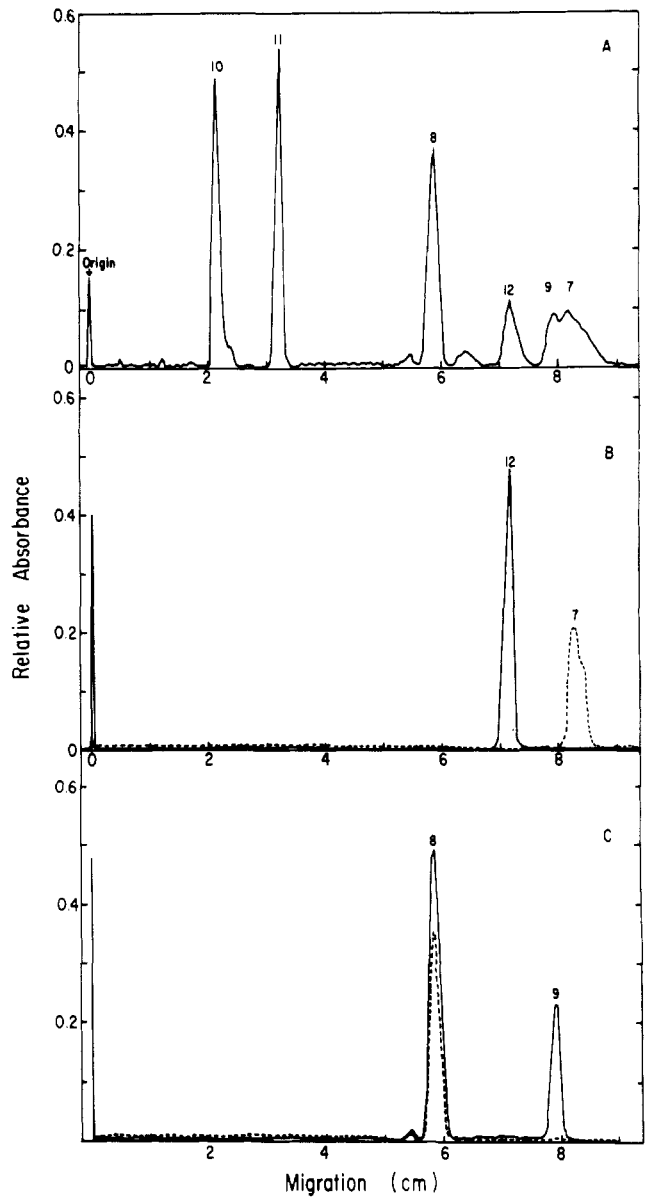

FIGURE 7: Identification of CNBr peptides by gel electrophoresis. (A) Total CNBr digest of partially hydroxylated protocollagen-P. (B) CB7 (---) and CB12 (—) from CM-cellulose chromatograph shown in Figure 6. (C) Pooled fraction of CB8 + CB9 from chromatograph in Figure 6 (—); CB8 after separation from CB9 by gel filtration (---).

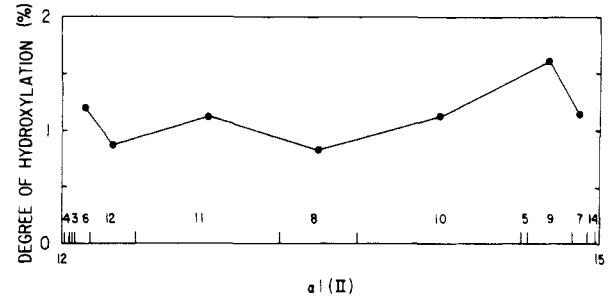

FIGURE 8: Corrected values for degree of hydroxylation of CNBr peptides of partially hydroxylated protocollagen-P from cells incubated with 0.15 M α,α'-dipyridyl.

100 triplets of -Gly-X-Pro in each pro α chain must be hydroxylated before the chains acquire enough hydroxyproline to fold into a triple helix which is stable at 37 °C [see Prockop et al. (1976, 1979) and Bornstein & Traub (1979)]. Prolyl hydroxylase must, therefore, interact with about 100 similar

substrate sites in a large polypeptide chain with a highly repetitive structure.

There are three general schemes by which the hydroxylations might proceed in terms of the order in which the triplets are hydroxylated. One is random hydroxylation in which all the -Gly-X-Pro- triplets are equivalent and have the same probabilities of being hydroxylated in the initial and subsequent encounters between enzyme and substrate. A second possibility is that the triplets vary in their reactivity based on the amino acids found in the X and Y positions around the residue which is hydroxylated but that triplets of high and low reactivity are widely distributed in the chain. As a result, initial hydroxylations might occur at a limited number of sites of high reactivity, and subsequent hydroxylations might proceed with an ordered or semiordered selection of sites based on the varying reactivities of different triplets. At all levels of hydroxylation, however, the amount of hydroxyproline in different regions of the chain would be about the same. A third possibility is that initial hydroxylation occurs in a specific region such as the amino- or carboxy-terminal end and that further hydroxylations proceed from this site in a linear fashion. This pattern of hydroxylation might occur if triplets of high reactivity were clustered in one region of the chain and adjacent regions contained triplets arranged on a decreasing gradient of reactivity. Alternatively, this pattern of hydroxylation might occur if the mechanism of the reaction were processive or "zipperlike" so that a linear pattern of hydroxylation was intrinsic to the enzymic reaction itself.

Two kinds of observations have ruled out the possibility that all -Gly-X-Pro- triplet pro α chains are equivalent and that prolyl hydroxylation of the chains is completely random. One observation is the variation which is seen in α(I) chains from rat and calf in terms of the degree to which prolyl residues in different triplets are hydroxylated (Bornstein, 1967; Bornstein & Traub, 1979). Such variation could not arise if all the triplets were equivalent during enzymic hydroxylation of the chains. More direct evidence for the nonequivalence of Gly-X-Pro- or -X-Pro-Gly- triplets comes from studies with synthetic peptides. Derivatives of bradykinin, which has a single triplet of -X-Pro-Gly-, were shown to vary widely in their K_m and V_{max} values, depending on amino acids introduced into the X position or into other positions closer to the amino-terminal end (McGee et al., 1973). Similarly, peptides of the structure $(X\text{-Pro-Gly})_5$ varied in K_m and V_{max} values when the X position was substituted with proline, glutamate, or arginine (Okada et al., 1972; Kivirikko et al., 1972; Prockop et al., 1976; Bhatnagar & Rapaka, 1976). Studies with the synthetic peptides $(\text{Pro-Pro-Gly})_5$ and $(\text{Pro-Pro-Gly})_{10}$ further demonstrated that identical triplets do not necessarily have the same reactivity but can be nonequivalent based on their position within the peptide. In the case of $(\text{Pro-Pro-Gly})_5$ the fourth triplet from the amino end was more readily hydroxylated than any other, and in the case of $(\text{Pro-Pro-Gly})_{10}$ the ninth triplet was preferentially hydroxylated (Berg et al., 1977). The preferential hydroxylation of the penultimate triplet in both of these peptides was apparently not based on proximity to the negative charge on the carboxy-terminal residue. Instead, the results suggested that the effect was explained by an ordered array of binding and reactive subsites within the enzyme itself.

The observations made here on the hydroxylation of protocollagen-P in vitro demonstrate that the enzymic reaction does not in itself produce preferential hydroxylation of any specific region of the chain. If a region such as the amino-terminal end had been preferentially hydroxylated, a gradient in the distribution of hydroxyproline should have been apparent in CNBr peptides from α chains that were hydroxylated so that on the average they contained 1.3–2.7 residues of hydroxyproline per chain. The results therefore indicated that the enzymic reaction does not involve any mechanism that makes it essential for hydroxylation to begin in any specific region of the chains. Initial hydroxylations must select the triplets that have the highest reactivity with prolyl hydroxylase, but the most reactive triplets are fairly uniformly distributed in the α(II) chains examined here.

The observations made with cells in which prolyl hydroxylase was partially inhibited with α,α'-dipyridyl demonstrate that the geometry imposed upon the enzyme and substrate by their location in cellular organelles also does not necessitate preferential hydroxylation of a specific region such as the amino-terminal region of the chains. Instead, it appears that when the prolyl hydroxylase activity in cells is low relative to the rate at which pro α chains are synthesized, hydroxylation of the chains proceeds in much the same pattern as is seen with isolated α chains and enzyme. The enzyme and the newly synthesized pro α and pro γ chains are therefore geometrically distributed within cellular organelles in a manner which allows continuing interactions of enzyme with all of the collagen domain of the protein. Such interactions with different regions of the collagen domain apparently proceed until the chains acquire enough hydroxyproline to fold into the triple-helical conformation which prevents further binding of enzyme to the protein (Kao et al., 1979).

The propeptides of procollagen were not examined here and, therefore, the results do not exclude the possibility that collagen-like domain in the type II amino-terminal propeptide (Curran et al., 1981) is preferentially hydroxylated compared to -Gly-X-Pro- triplets in the α-chain domain. However, even if such a collagen-like sequence in the propeptide is preferentially hydroxylated, most of the hydroxyproline required for formation of the triple helix is apparently introduced into the molecule in the nonpreferential pattern of hydroxylation observed here.

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Metal Ion Binding to α-Lactalbumin Species†

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ABSTRACT: A strong cation (calcium) binding site has been demonstrated to exist in several α-lactalbumin species: bovine, goat, human, and guinea pig. A metal ion induced conformational change occurs, resulting in a unique (10–14-nm) blue shift and relative quenching of Trp fluorescence for all species. Calcium ion binding to the α-lactalbumins yielded dissociation constants (K_{diss} consistently in the 10^{-10}–10^{-12} M range, while Mn(II) binding was in the 20–30 μM range. Independent determinations of these cation binding equilibria were made by ESR measurements of free unliganded Mn(II) in titrations with the bovine species. One strong site (K_{diss} = 30.5 μM) was found, which correlated directly with the fluorescence-associated cation binding, plus three weaker sites (K_{diss} = 1.1, 5.0, and 5.0 mM, respectively). Several lanthanides as well as Mg(II) were found to displace Mn(II) from the strong site on bovine α-lactalbumin (as monitored by ESR) and to cause the identical fluorescence changes as found for Ca(II) and Mn(II) above. The importance of measuring these equilibria by both fluorescence and ESR was borne out by demonstrating the potential errors in estimating dissociation equilibria by the fluorescence method alone. Also, the errors in estimating K_{diss} for samples containing partially metal bound apo-α-lactalbumin are described as well as rapid, sensitive methods for estimating the extent of metal-free protein and correctly accounting for residual bound metal in equilibrium calculations.

α-Lactalbumin (αLA)[1] serves as a "modifier" protein in lactose biosynthesis ("lactose synthase") as the noncatalytic subunit in complex with galactosyltransferase (UDP-galactose:D-glucose 4-β-D-galactosyltransferase, EC 2.4.1.22). The metal binding properties of bovine αLA (BLA) were discovered in our laboratory by ESR and NMR as part of control experiments for Mn(II) binding to the catalytic subunit of the lactose synthase complex (Berliner et al., 1978; Andree & Berliner, 1980). Both Brittain et al. (1976) and Hiraoka et al. (1980) presented evidence for Tb(III) and Ca(II) binding to bovine αLA, respectively, but no equilibrium data were presented nor specific evidence for the exact binding locus. Precise determination of cation–αLA equilibrium binding constants and stoichiometry [for, e.g., Mn(II), Tb(III), etc.] is crucial to NMR, ESR, and fluorescence measurements of intermolecular distance relationships in the physiologically significant lactose synthase complex.

We have also been involved in characterization of structural homologies in a series of αLA species (Berliner & Kaptein, 1981) and have addressed whether these metal ion binding phenomena are common to most α-lactalbumins as a general structural feature. We present fluorescence and ESR measurements of the binding equilibria for several cations [Ca(II), Mn(II), Cd(II), Mg(II), lanthanides] with bovine, goat, human, and guinea pig α-lactalbumins. A subsequent paper will describe NMR evidence for the specific amino acid residue(s) affected by this cation binding, in particular that region adjacent to histidine-68 in bovine and goat α-lactalbumins (H. Nishikawa, K. Murakami, and L. J. Berliner, unpublished experiments).

Experimental Procedures

Proteins. Electrophoretically pure bovine αLA (lots 75C 8110, 86C 8020, and 50F 8105) was from Sigma Chemical Co. Other αLA species were obtained or isolated as noted

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[1] Abbreviations: αLA, α-lactalbumin; BLA, bovine α-lactalbumin; GLA, goat α-lactalbumin; HLA, human α-lactalbumin; GPLA, guinea pig α-lactalbumin; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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