Affinity Column Purification of Protocollagen Proline Hydroxylase from Chick Embryos and Further Characterization of the Enzyme*

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SUMMARY

An affinity column procedure was devised for the purification of protocollagen proline hydroxylase, the enzyme which synthesizes the hydroxyproline in collagen by hydroxylating peptidyl proline. The procedure involved linking a peptide substrate with a high affinity for the enzyme to agarose and then specifically eluting the enzyme from the agarose with a high concentration of a second peptide substrate of lower affinity. The second substrate was then separated from the enzyme by gel filtration. The isolated enzyme was homogeneous by gel filtration, by ultracentrifugation of the enzyme in the native and dissociated state, and by polyacrylamide gel electrophoresis in the native and dissociated state. Also, the enzyme had a considerably higher specific activity than enzyme isolated from the same source with other procedures.

The enzyme was shown to be a tetramer which has a molecular weight of about 230,000 and is composed of two different types of subunits. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate indicated that the molecular weights of the two types of subunits were 60,000 and 64,000. Ultracentrifugation of the enzyme in guanidine hydrochloride and mercaptoethanol indicated that the molecular weight for the 2 subunits was 62,000. The enzyme was dissociated into both monomers and dimers by either dithiothreitol or mercaptoethanol, indicating that the structural integrity of the enzyme is maintained in part by either intrachain or interchain disulfide bonds. The monomers had no enzymatic activity.

The isolated enzyme was shown to have no protocollagen lysine hydroxylase activity. All of the hydroxyproline synthesized by the enzyme was shown to be 4-hydroxyproline, and no 3-hydroxyproline activity was detected.
MATERIALS AND METHODS

Materials—Chick embryos from white Leghorn chickens were purchased from Shaw Hatcheries (West Chester, Pa.) at the age of 12 days, and they were incubated in a moist atmosphere at 37°C for 24 hours. RCM from the cuticle of Ascaris lumbricoides var. suis was prepared according to the procedure of McBride and Harrington (15) from live worms which were obtained from pig intestines at local slaughterhouses in Philadelphia, Pennsylvania. The worms were frozen and thawed several times to loosen the cuticles which were then removed by dissection, minced, and washed in 1 liter of 0.5 M NaCl for 2 hours. About 245 g (wet weight) of the cuticles were homogenized in 500 ml of 0.5 M NaCl in a Sorvall high speed Omnimixer. The homogenates were extracted three times with 2 liters of cold 0.5 M NaCl for 24 hours, and the extracts were discarded. The residue remaining after the NaCl extraction was reduced with 1% mercaptoethanol (Eastman) in the presence of 5 M guanidine hydrochloride (Sigma) and then carboxymethylated with monooiodoacetic acid (15).

(Pro-Gly-Pro), with an average molecular weight of about 4200 was purchased from Miles-Yeda, Ltd., Rehovoth, Israel. Protocollagen labeled with [14C]proline was prepared with cartilage from chick embryos as described previously (12), and aliquots of 10 μg per ml were stored frozen in volumes of 1 ml.

Preparation of the Affinity Column—in order to prepare the affinity column, substrate was covalently bound to agarose by previously published techniques (16). Two hundred milliliters of 6% agarose (Bio-Gel A-5m, 100 to 200 mesh, Bio-Rad) were diluted with 200 ml of distilled water at 4°C, and the pH was adjusted to 11 with dilute NaOH. Fifty grams of cyanogen bromide (Eastman) were added, and the pH was readjusted to 11 with 8 N NaOH. As the activation of the gel proceeded, the pH was maintained at 11 by addition of 8 N NaOH, and the temperature was kept below 20°C by adding ice. After 10 min, the agarose was washed in a Buchner funnel with 2 liters of 0.14 M NaCl and 0.1 M NaHCO3 (pH 9.3). The coupling reaction was carried out by quickly stirring 2.4 g of RCM in 240 ml of buffer into the gel on the funnel. The sample was immediately transferred to a beaker and stirred at 4°C for 20 hours. The product was washed first with 2 liters of 1 M NaCl and 0.2 M sodium borate (pH 8.25) and then with 2 liters of 1 M NaCl and 0.1 N acetic acid. In order to measure the efficiency of the coupling reaction, an aliquot of the gel was hydrolyzed in 6 N HCl at 120°C for 15 hours and assayed on an amino acid analyzer. Micromoles of aspartic acid, glycine, and proline were used to calculate the amount of RCM contained in 1 ml of packed gel.

Preparation of the Ammonium Sulfate Fraction of Chick Embryo Homogenate—Two hundred 13-day-old chick embryos (1.3 kg, wet weight) were homogenized in an equal volume of 0.01 M KCl. The homogenization was carried out in batches of 30 embryos at high speed on a Waring Blender for 60 s. The homogenate was centrifuged at 15,000 × g for 30 min, and the pellet was discarded. The supernate was precipitated by adding solid ammonium sulfate (Baker Chemical Company) to a saturation of 30% (17.6 g per 100 ml). The pellet was removed by centrifugation at 15,000 × g for 30 min, and the supernate was precipitated with solid ammonium sulfate at a concentration of 65% saturation (23.5 g per 100 ml). The pellet was again removed by centrifugation at 15,000 × g for 30 min and dissolved in 0.05 M KCl, 0.1 M NaCl, 0.1 M glycine, and 0.01 M Tris-HCl buffer adjusted to pH 7.8 at 4°C. The ammonium sulfate fraction in a volume of about 1 liter was dialyzed for 12 hours against 4 liters of the buffer and then for 6 hours against another 4 liters of buffer. The sample was adjusted to a protein concentration of 15 mg per ml with buffer, and it was stored frozen in aliquots of 300 ml.

Purification of the Enzyme on the Affinity Column—the agarose coupled with RCM was poured into a glass column (1.5 × 30 cm) with a bed volume of 50 ml. The gel was equilibrated with "enzyme buffer" which consisted of 0.2 M NaCl, 0.2 M glycine, 10 μM dithiothreitol (Sigma), and 0.01 M Tris-HCl buffer adjusted to pH 7.8 at 4°C.

Three hundred milliliters of the ammonium sulfate fraction with a protein concentration of approximately 15 mg per ml were thawed and centrifuged at 20,000 × g for 40 min in order to remove a small amount of insoluble protein. The sample was diluted with the enzyme buffer to a concentration of 7.5 mg per ml and then passed through the affinity column at a flow rate of about 50 ml per hour. The column was washed with approximately 500 ml of enzyme buffer until the optical density at 230 nm of the eluate was less than 0.1. The enzyme was then eluted from the column with 30 ml of buffer containing 10 mg per ml of (Pro-Gly-Pro)α followed by 30 ml of enzyme buffer.

The fractions containing the enzyme eluted with the (Pro-Gly-Pro)α were pooled and placed on a 6% agarose column (Bio-Gel A-5m 200 to 400 mesh, Bio-Rad). The column was 2.5 × 90 cm, and the sample volume was approximately 30 ml. The column was equilibrated and eluted with enzyme buffer, and fractions of 5.3 ml were collected. The fractions containing the enzymatic activity were concentrated by ultrafiltration with an Amicon ultrafiltration cell (model no. 52) with a PM30 membrane. All these procedures were carried out at 4°C, and the enzyme was stored frozen.

Assay of Enzymatic Activity—The enzyme was assayed by either one of two procedures. In order to assay the enzyme in impure fractions, (Pro-Gly-Pro)α was used as a substrate, and the amount of hydroxyproline synthesized was measured with a specific chemical procedure (17). With purified preparations of enzyme, the assay was carried out with RCM as a substrate and by measuring the release of 14CO2 from α-keto[1-14C]glutaric acid (18).

When the enzyme was assayed by the quantitative synthesis of hydroxyproline, the reaction was carried out in a final volume of 4 ml which contained 5 to 15 units of enzyme, 250 μg of (Pro-Gly-Pro)α per ml, 2 mg of bovine serum albumin (Sigma) per ml, 0.1 mg of catalase (Calbiochem) per ml, 0.05 mM FeSO4, 2 mM ascorbate, 0.5 mM α-ketoglutaric acid (Calbiochem), 0.1 mM diithiothreitol, and 0.05 M Tris-HCl buffer (pH 7.8) at 37°C (6). The sample was incubated at 37°C for 1 hour and then hydrolyzed in 6 N HCl at 120°C for 24 hours. The hydrolyzed sample was evaporated in a rotary evaporator and assayed for hydroxyproline (17). [14C]Proline-labeled protocollagen was used as a substrate, the sample was treated as above except that [14C]hydroxyproline was assayed by a different chemical procedure (19).

In experiments in which the enzyme was assayed by the evolution of 14CO2 (18) the reaction was carried out in a final volume of 1.0 ml which contained 1 to 5 units of enzyme and the same concentration of co-factors as described above for the
quantitative production of hydroxyproline, except that RCM was used as the substrate and α-ketoglutarate was included. The RCM concentration was 75 μg per ml or 7.5 times its K_m. The RCM was boiled at 100°C for 10 min and quenched to 4°C immediately before addition to the incubation system. α-Ketoglutarate (Calbiochem) with a specific activity of 23.6 mCi per mM was diluted with cold α-ketoglutarate (Calbiochem) to give a specific activity of 240 cpm per pmole. One hundred nanomoles of α-ketoglutarate or 24,000 cpm was added per ml of reaction mixture. The samples were incubated at 37°C for 20 min, and the 14CO_2 was counted in an Intertechnique liquid scintillation counter at an efficiency of 92% for 14C.

Definition of Enzyme Units—One unit of enzymatic activity was defined as the amount of enzyme required to synthesize 1 μg of hydroxyproline in 1 hour under conditions in which the concentrations of co-factors and co-substrates were saturating, and 250 μg of (Pro-Gly-Pro)_n per ml was used as substrate. With the preparations of the polymer used here, 250 μg per ml was 58% of the saturating concentration. Experiments in which the same enzyme preparation was assayed with both the quantitative synthesis of hydroxyproline and with the evolution of 14CO_2 from α-ketoglutarate indicated that 1 unit of enzymatic activity produced the release of 670 cpm of 14CO_2 per min. This value is about 30% less than the value which was predicted from the specific activity of the α-ketoglutarate, an observation which is explained by the fact that the commercially available α-ketoglutarate is only 70% pure.

The protein content of various samples was assayed either by absorbance at 230 nm with standards of serum albumin or by ninhydrin assays after hydrolysis of the samples. The extinction coefficient with a 1-cm light path, E^1 cm^1 per mg, was 4.95 for the enzyme protein, and it was 3.25 for the (Pro-Gly-Pro)_n.

Disc Electrophoresis on Polyacrylamide Gels—Polyacrylamide gel electrophoresis was performed in three systems. The electrophoresis of native protein was carried out with 7.5% polyacrylamide at 4°C with 42.5 mM Tris-HCl and 45.3 mM glycine buffer adjusted to pH 6.6 at 0°C as the upper buffer. The lower buffer was 120 mM Tris-HCl adjusted to pH 8.84 at 0°C. The enzyme sample was applied directly to the polyacrylamide gel without the addition of sucrose since preliminary experiments indicated the enzyme tended to dissociate in the presence of sucrose (see below). Electrophoresis was carried out in a Buchler Polyanalyst temperature-regulated apparatus, and the gels were stained with Coomassie blue.

In order to carry out the electrophoresis on the denatured polypeptide chains, the method of Weber and Osborn (21) was used with 10% polyacrylamide gels in 0.1% sodium dodecyl sulfate and 0.2 M sodium phosphate (pH 7.2). The denatured polypeptide chains were also examined by incubating the enzyme in 6 M urea and 0.1 M mercaptoethanol and then subjecting them to electrophoresis under the same conditions used for the native enzyme (see above).

Determination of Molecular Weight by Analytical Ultracentrifugation—The molecular weight of the native enzyme was determined by using equilibrium sedimentation according to the method of Yphantis (22) in a Beckman model E analytical ultracentrifuge employing Rayleigh optics. All experiments were performed with double-sector synthetic boundary cells fitted with sapphire windows. The samples were prepared by concentrating the enzyme in enzyme buffer with an ultracentrifuge and using the ultracentrifuged as reference solution. In the two experiments, the sample volumes were 170 and 140 μl, and ultracentrifugates of 240 and 250 μl, respectively, were placed in the reference cell. In the first experiment a protein concentration of 664 μg per ml was employed, the column height was 5 mm, and the centrifugation was at 13,000 rpm for 24 hours at 16.7°C. In a second experiment the protein concentration was 1.3 mg per ml, the column height was 480 mm, and the centrifugation was at 10,000 rpm at 13.8°C. In the second experiment photographic plates were obtained at 24 and 36 hours, and measurements of the fringe displacements indicated that equilibrium was attained at 24 hours. In both experiments the conditions were adequate to deplete the meniscus. The weight average molecular weight was calculated from the slope of a plot of the log fringe displacement versus the square of the radius of rotation. Five measurements of fringe displacement were made at each radial position, and the uncertainty in determining fringe displacement was about 2 μm, corresponding to 0.01 fringe. In all cases the slope was determined by calculation of the linear regression line. A partial specific volume of 0.74 was calculated (23) from the amino acid composition.

In order to measure the molecular weight of the subunits of the enzyme, the protein solution was dialyzed exhaustively against 5.8 M guanidine hydrochloride (Schwarz/Mann) and 0.1 M mercaptoethanol. The dialysate was used as the reference solution. The molecular weight was determined in the same manner as was used for the native enzyme (above), except that the rotor speed was 20,000 rpm and the temperature was 20°C. The column height was 5.4 mm, the time of centrifugation was 24 hours, and the rotor speed was again adequate to deplete the meniscus. The molecular weight was calculated as described above except that a Φ of 0.73 was used (24).

Amino Acid Analysis of the Enzyme—The enzyme was dialyzed exhaustively against distilled water, oxidized with performic acid (25), and then hydrolyzed in 6 N HCl for 24 hours in tubes sealed under N_2. The sample was then evaporated in a rotary evaporator on a boiling water bath to dryness and dissolved in 1 ml of the standard citrate buffer used for amino acid analysis. Amino acid analysis was performed with a Beckman model 116 amino acid analyzer.

EXPERIMENTAL RESULTS

Purification of the Enzyme by Affinity Chromatography—The substrate RCM was covalently bound to the agarose gel by the procedure of Porath as described by Cuatrecasas (16). After the coupling reaction was carried out at pH 8.1, amino acid analysis of the gel indicated that about 1.44 mg of RCM was coupled per ml of the packed gel. When the coupling reaction was carried out at pH 9.3, about 4.63 mg of RCM was coupled per ml of packed gel. RCM coupled to agarose at pH 9.3 was used to make the affinity column described below.

Previous results indicated that fractionalation of a homogenate of chick embryos with 30 to 65% saturated ammonium sulfate
gave a crude enzyme preparation whose specific activity was approximately three times that of the 15,000 x g supernate of the homogenate (26). When this ammonium sulfate fraction was passed through the affinity column, over 99% of the protein was directly recovered in the column effluent but the column effluent contained only 6 to 10% of the total enzymatic activity of the sample applied to the column (Fig. 1). After the column was washed with buffer, the enzyme was eluted by passing through a solution containing 10 mg of (Pro-Gly-Pro)$_3$ per ml. Direct assay of the eluate indicated that about 55% of the enzyme applied to the column was recovered (Table I). There was only an 8-fold increase in specific activity, but most of the protein in the fraction was accounted for by the (Pro-Gly-Pro)$_3$, which was used to elute the column. The (Pro-Gly-Pro)$_3$ was then separated from the eluted enzyme by pooling the appropriate fractions and chromatographing them directly on a 6% agarose column (Fig. 2). Enzymatic activity was eluted well ahead of the polymer, and essentially all of the enzyme placed on the column was recovered. The over-all recovery of enzyme through the chromatographic steps was 58%. The peak of enzymatic activity eluted from the agarose column was coincident with the small peak of protein eluted in the same position and the specific activity was 2,710, or 1,600-fold greater than that of the original ammonium sulfate fraction (Table I). Similar results were obtained in six additional experiments (not shown).

Further experiments demonstrated that the fractions containing enzyme activity (Fig. 2) did not contain measurable amounts of the (Pro-Gly-Pro)$_3$ used to elute the affinity column. Fifty microliters of Fractions 65 to 76 (Fig. 2) were incubated under the standard conditions for the a-keto[14C]-glutarate assay for enzyme activity (see "Materials and Methods") except that no peptide substrate was added. No enzymatic activity was observed under conditions in which activity would have been detected if 3% of the protein in the peak fractions (Fraction 69) had been (Pro-Gly-Pro)$_3$.

**Purity of the Enzyme—Polyacrylamide gel electrophoresis of the purified enzyme in Tris-glycine buffer at pH 9.6 indicated that most of the protein was recovered in a single band in the upper part of the gel (Gel A in Fig. 3). Two minor bands were seen further down in the gel but subsequent experiments demonstrated that both of these bands were derived from the major band of the enzyme protein (see below). No other stained bands were seen even when the amount of enzyme applied to the gel was as much as 40 µg or about 40 times the amount of protein required to detect the major band of enzyme protein with the Coomassie blue stain. Also, there was no evidence of contaminating protein when the gels were stained with Amido schwarz, and there was no stained material in the stacking gel or at the origin in any of the experiments.**

**Molecular Weight of the Enzyme**—The molecular weight of the native enzyme was estimated by placing a sample of enzyme on an 8% agarose (Bio-Gel A-1.5m) column which had been calibrated with proteins of known molecular weights. The results indicated an apparent molecular weight of 350,000. The molecular weight was also measured by equilibrium sedimentation in an analytical ultracentrifuge at both 10,000 and 13,000 rpm. The plot of the log of the fringe displacement versus $r^2$ was linear across the cell. The calculated molecular weight of the enzyme was 250,000 for the run which was carried out at 10,000 rpm and 227,000 for the run which was carried out at 13,000 rpm. From the 95% confidence interval of the slopes of the plots, it was estimated that the precision of the measurements was ±5,000 daltons. Since the values for molecular weight at two speeds and two different concentrations were about the same, the results suggested that the measurements were not affected by thermodynamic non-ideality.

**Dissociation of the Enzyme into Subunits**—The enzyme could...
be partially dissociated by exposing it to higher concentrations of dithiothreitol than were present in the buffer used to prepare the enzyme, by exposing it to buffers of low ionic strength, or by exposing it to 6 M urea and 0.1 M mercaptoethanol. The enzyme which was prepared in 0.2 M glycine, 0.2 M NaCl, 0.01 M Tris-HCl (pH 7.8), and 0.01 M dithiothreitol was incubated at room temperature for 3 hours after raising the dithiothreitol concentration to 1 mM. The treatment decreased the enzymatic activity by 90%, and polyacrylamide gel electrophoresis indicated that the major band became much fainter, whereas the minor bands increased in intensity (Gel B in Fig. 3). Most of the protein was recovered in the band with the highest mobility, and close examination of this band indicated it consisted of two bands of equal intensity (Fig. 3). In a control experiment, enzyme which was incubated at room temperature for 3 hours without the increased concentration of dithiothreitol showed no loss of enzyme activity and no evidence of dissociation when examined by polyacrylamide electrophoresis.

The enzyme was also dissociated by dialyzing it for 24 hours at 4°C against a low ionic strength buffer consisting of 10 mM Tris-HCl (pH 7.8) and 10 μM dithiothreitol. The treatment decreased the enzyme activity by 40%, and polyacrylamide gel electrophoresis indicated that about one-half of the protein in the major band had been dissociated (Gel C in Fig. 3). In this case most of the dissociated protein was recovered in the band of intermediate mobility.

More complete dissociation of the enzyme was obtained by incubating the enzyme with 6 M urea and 0.1 M mercaptoethanol at 37°C for 1 hour. Polyacrylamide gel electrophoresis indicated that all of the protein was obtained in two bands of high mobility (Gel D in Fig. 3) corresponding to the two kinds of subunits of the enzyme (see below).

Attempts to subject the enzyme to electrophoresis at pH values lower than its isoelectric point of 4.4 (6) were unsuccessful because the enzyme formed insoluble aggregates, even in the presence of 6 M urea and 0.1 M mercaptoethanol.

To determine whether the minor bands of protein (Fig. 3) were enzymatically active, the enzyme was dialyzed for 24 hours at 4°C against the low ionic strength buffer (above) and subjected to polyacrylamide gel electrophoresis. The gel was sliced, the slices were homogenized and then assayed for pro-collagen proline hydroxylase by incubating them with [14C]proline-labeled pro-collagen in a 4.0-mL total volume containing the necessary co-factors and assaying for [14C]hydroxyproline (19). Although the treatment with the low ionic strength buffer dissociated about one-half of the enzyme to a form which has an intermediate mobility, the band in the upper part of the gel still accounted for about 88% of the enzymatic activity recovered from the gel (Fig. 4). A small amount of activity was detected in the intermediate band, but the results suggested that the specific activity of this protein was no more than one-seventh that of the protein in the upper band. No significant activity was found in the bands of highest mobility. We interpret the observations to indicate that the major slow moving band obtained with the isolated enzyme is a tetramer form, the band of intermediate mobility obtained after partial dissociation is a dimer, and the split band of highest mobility consists of the two types of monomers found in the enzyme (see below).

Subunit Structure of the Enzyme—It was found that the enzyme could be dissociated by treating it with SDS and mercaptoethanol. Examination of the dissociated enzyme by polyacrylamide electrophoresis in SDS (Fig. 5) indicated that all of the protein was recovered in two bands which were of equal intensity and which had very similar RF values. There was no evidence of any other protein either in the gels or at the origin. Calibration of the SDS gels with standard proteins indicated that the molecular weights of the two polypeptide chains as estimated by this technique were 64,000 and 60,000.

![Fig. 3. Polyacrylamide gel electrophoresis of purified enzyme and partially dissociated enzyme. The electrophoresis was carried out at 42.5 mM Tris-glycine buffer, (pH 9.6), as described in "Materials and Methods." The anode is at the bottom. The stacking gel is seen on Gels B, C, and D but is not present on Gel A. The gels were run at separate times, and gels were cut at the buffer front. Gel A, enzyme prepared by affinity column chromatography; Gel B, enzyme partially dissociated by increasing the dithiothreitol concentration of the enzyme buffer from 0.01 to 1 mM. Gel C, enzyme partially dissociated by dialyzing it against a low ionic strength buffer; Gel D, enzyme completely dissociated by incubating it with urea and mercaptoethanol.](http://www.jbc.org/)

![Fig. 4. The location of pro-collagen proline hydroxylase activity after polyacrylamide gel electrophoresis of partially dissociated enzyme. The enzyme was dialyzed against a low ionic strength buffer as described in the text, and the bands of protein detected with the Coomassie blue are indicated along the bottom of the graph (compare with Gel C in Fig. 3). The anode is on the right. The disc was sliced into 3.5-mm slices and incubated with [14C]proline-labeled pro-collagen and assayed as described in the "Materials and Methods." Each bar in the graph indicates the hydroxyproline formed by enzyme contained in a slice of gel.](http://www.jbc.org/)
The molecular weight of the subunits of the enzyme was also measured by sedimentation equilibrium in the presence of 5.8 M guanidine HCl and 0.1 M mercaptoethanol. The plot of log fringe displacement versus r^2 was linear because the 2 subunits of the enzyme have very similar molecular weights. Calculations of the molecular weight of the subunits by using the slope of a line through the data points gave a molecular weight of 62,000. From the 95% confidence interval of the slope, it was estimated that the precision of the measurement was about ±1,000 daltons.

**Amino Acid Analysis of the Enzyme**—About 0.8 mg of enzyme was isolated by affinity column chromatography as described above (Figs. 1 and 2), concentrated, and rerun on a second agarose column (not shown) in order to remove any of the (Pro-Gly-Pro), used to elute the enzyme which may still be bound to the enzyme. All of the protein recovered from the column was in the enzyme peak. The enzyme was then dialyzed exhaustively against distilled water to remove the glycine used in the enzyme buffer. The final yield of enzyme was about 0.5 mg. One aliquot of the protein was hydrolyzed directly in HCl, and another aliquot was oxidized with performic acid and then hydrolyzed in HCl (25). Amino acid analysis of the hydrolysate gave results which differed slightly from those reported previously for protocollagen proline hydroxylase (Table II). The most prominent difference from the amino acid composition reported by Rhoads and Udenfriend (7) for the proline hydroxylase from newborn rat skin was that it contained 28 residues per 1,000 less of serine. The most prominent differences from the values reported by Halme et al. (5) for the enzyme from chick embryos were that it contained, per 1,000 residues, 62 residues less of serine, 43 residues less of glutamic acid, and 43 residues more of leucine. The difference in amino acid analysis between the results obtained here and those reported by Halme et al. (5) are in part explained by the fact that the enzyme prepared by Halme et al. contained a minor contaminant and only a limited amount of protein was available for analysis. The differences from the results reported by Rhoads and Udenfriend (7) may be explained either by a species difference in the structure of the enzyme or by the fact that the enzyme prepared by Rhoads and Udenfriend (7) contained several minor contaminants.

**Absence of Protocollagen 3-Proline Hydroxylase Activity and of Protocollagen Lysine Hydroxylase Activity**—To determine whether the purified enzyme could synthesize 3-hydroxyproline as well as 4-hydroxyproline, [14C]proline-labeled protocollagen from chick cartilage was incubated with excess enzyme for 1 hour. The incubation with enzyme converted 38% of the [14C]proline in the protocollagen to 4-hydroxy[14C]proline but no significant amount of 3-hydroxy[14C]proline was synthesized (Table III). Previous studies (27) with [14C]proline-labeled protocollagen prepared in the same manner indicated that about 65% of the [14C] in the preparation was [14C]proline in protocollagen and about 35% was [14C]proline in noncollagenous proteins. On this basis, the incubation with the enzyme had fully hydroxylated the susceptible [14C]proline residues in the protocollagen, but no 3-hydroxy[14C]proline was synthesized.

Further experiments demonstrated that the purified enzyme did not contain significant amounts of protocollagen lysine hydroxylase activity. [14C]Lysine-labeled protocollagen was prepared from chick embryo cartilage and was used as substrate for the synthesis of hydroxy[14C]lysine under the conditions described previously (20, 28). The ammonium sulfate fraction used as the starting material for affinity chromatography had a specific activity of 1 to 1.5 as defined

| Amino acid | Values from Rhoads and Udenfriend (7) | Values from Halme et al. (5) | Values obtained here* |
|------------|-------------------------------------|-----------------------------|----------------------|
|            | residues/1000 residues               | residues/1000 residues      | residues/1000 residues |
| Lysine     | 77                                   | 85                          | 93                   |
| Histidine  | 19                                   | 25                          | 17                   |
| Arginine   | 44                                   | 35                          | 54                   |
| Cysteic acid | 12                               | 35                          | 12                   |
| Aspartic acid | 107                              | 95                          | 90                   |
| Methionine sulfoxide | 11                           | 15                          | 7                    |
| Threonine  | 57                                   | 50                          | 46                   |
| Serine     | 66                                   | 100                         | 38                   |
| Glutamic acid | 149                             | 180                         | 137                  |
| Proline    | 50                                   | 35                          | 50                   |
| Glycine    | 69                                   | 100                         | 82                   |
| Alanine    | 83                                   | 75                          | 90                   |
| Valine     | 63                                   | 50                          | 58                   |
| Isoleucine | 40                                   | 35                          | 44                   |
| Leucine    | 95                                   | 65                          | 108                  |
| Tyrosine   | 28                                   | 20                          | 11                   |
| Phenylalanine | 42                            | 30                          | 57                   |

* Enzyme purified from newborn rat skin.

† Enzyme purified from chick embryos.

Values are the mean of duplicate analyses in which the two values for any given amino acid did not differ by more than 6 residues per 1,000 residues. Tryptophan was not determined here or in previous analyses (5, 7).

K. I. Kivirikko, personal communication.
The enzyme was incubated with $[^3]C$proline-labeled procollagen as described in “Materials and Methods” for 1 hour at 37°C. The medium was then dialyzed against water to remove buffers, hydrolyzed in 0.1 M HCl, evaporated, and applied to the long column of an amino acid analyzer. Fractions were collected and counted in a scintillation counter, and the elution positions of the 3-hydroxy-[^3]Cproline and 4-hydroxy[^3]Cproline peaks were identified with standards in a separate run.

The amount of $[^3]C$proline-labeled procollagen used in Experiment 1 was $7.7 \times 10^{10}$ cpm, and in Experiment 2 it was $88.9 \times 10^{10}$ cpm.

Previous results demonstrated that over 90% of the $[^3]C$ in procollagen was $[^3]C$proline (27). Residues per α chain were calculated from the ratio of 4-hydroxy[^3]Cproline synthesized to the total amount of $[^3]C$proline plus $[^3]C$hydroxyproline in the substrate after hydroxylation. This has been referred to as percentage of hydroxylation (27). The total amount of hydroxyproline and proline in chick tendon used in these calculations for converting percentage of hydroxylation into residues of α chain was obtained from Eastoe (31). Previous studies (27) with [^3]Cproline-labeled procollagen indicated that 35% of the $[^3]C$proline was in noncollagen proteins, and values of residues per 1000 residues were adjusted accordingly.

by Kivirikko and Prockop (29, 38). The purified procollagen proline hydroxylase eluted from the affinity column had a specific activity of less than 0.1 in terms of lysine hydroxylase activity, although the specific activity of the procollagen proline hydroxylase had been increased 1,600-fold.

### DISCUSSION

The purification of procollagen proline hydroxylase by the affinity column procedure described here was more efficient than most such procedures for enzyme purification for two reasons. First, procollagen proline hydroxylase has an extremely high affinity for large polypeptide substrates; the affinity constant ($K_{\text{aff}}$) for procollagen has been estimated to be greater than $10^{19}$ M$^{-1}$ (14). For this reason, crude extracts could be passed through the column, and the enzyme was efficiently retained. Second, the enzyme was eluted with a synthetic peptide which was essentially uncharged and therefore did not elute contaminating proteins which might be bound to the column by ionic forces. The eluting peptide was readily separated from the enzyme by gel filtration.

The enzyme obtained with the affinity column procedure was homogeneous by gel filtration, ultracentrifugation, and polyacrylamide gel electrophoresis in its native state. In addition, after the enzyme was completely dissociated and subjected to electrophoresis in the presence of either urea or SDS, only two bands of protein were obtained and these corresponded to the two kinds of subunits. More recently, the isolated enzyme was used to immunize rabbits, and antisera from the rabbits was found to contain high titers of antibodies which were specific as tested by immunodiffusion and immunoelectrophoresis (29).

### TABLE III

**Hydroxylation of[^3]Cproline-labeled procollagen with purified enzyme and assay for 4-hydroxy[^3]Cproline and 3-hydroxy[^3]Cproline**

| Experiment | Enzyme | E:S ratio$^b$ | 4-Hydroxy[^3]C-proline | 3-Hydroxy[^3]C-proline |
|------------|--------|------------|-----------------|-----------------|
|            |        | $\mu$g/$\mu$g $\times 10^4$ | $\mu$g | cpm | Residues per α chain$^a$ | $\mu$g | cpm | Residues per α chain$^a$ |
| 1          | None   | 3.0        | 105             | $<0.3$          |
|            |        | 6.7        | 7,700           | 99              |
| 2          | None   | 128.0      | 410             | $<0.2$          |
|            |        | 1.44       | 248,000         | 99              |

$^a$ The amount of $[^3]C$proline-labeled procollagen used in Experiment 1 was $7.7 \times 10^{10}$ cpm, and in Experiment 2 it was $88.9 \times 10^{10}$ cpm.

$^b$ Previous results demonstrated that over 90% of the $[^3]C$ in procollagen was $[^3]C$proline (27). Residues per α chain were calculated from the ratio of 4-hydroxy[^3]Cproline synthesized to the total amount of $[^3]C$proline plus $[^3]C$hydroxyproline in the substrate after hydroxylation. This has been referred to as percentage of hydroxylation (27). The total amount of hydroxyproline and proline in chick tendon used in these calculations for converting percentage of hydroxylation into residues of α chain was obtained from Eastoe (31). Previous studies (27) with $[^3]C$proline-labeled procollagen indicated that 35% of the $[^3]C$proline was in noncollagen proteins, and values of residues per 1000 residues were adjusted accordingly.

Päkkäinen et al. (6) reported a molecular weight of 248,000 by equilibrium sedimentation and 350,000 by gel filtration, values very similar to those obtained here. After dissociation of the enzyme, they found subunits of 110,000 for procollagen proline hydroxylase isolated from chick embryo homogenates. Rhoads and Udenfriend (8), on the other hand, reported a molecular weight of 109,000 to 148,000 for the enzyme isolated from newborn rat skin and molecular weights of 60,000 and 64,000 by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The difference between the molecular weight of the native enzyme obtained by gel filtration and by sedimentation equilibrium suggests that the enzyme is somewhat asymmetric, an observation consistent with data recently reported by Päkkäinen et al. (6).
entirely possible that they may have isolated a partially disso-
ciated form of the enzyme. Also, it seems probable that Päkkäläinen et al. (6) did not completely dissociate their en-
zyme with a molecular weight of 248,000 into the polypeptide
subunits, since only 0.1 mM dithiothreitol was used in their
sedimentation equilibrium studies with 5.8 M guanidine HCl,
whereas 100 mM mercaptoethanol was used in our experiment.3

The results obtained by hydroxylation of [14C]proline-labeled
protocollagen 3-proline hydroxylase activity under condi-
tions in which it converted essentially all of the susceptible
enzyme may be involved in synthesizing the small amount of
Piinkalainen et al. (6) did not completely dissociate their en-
tire form of the enzyme. Also, it seems probable that
they may have isolated a partially disso-
ciated form of the enzyme. Also, it seems probable that

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3 We previously reported (32) that protocollagen proline hy-
droxylase prepared by the procedure of Halme et al. (5) consisted
either of single ring structures with a molecular weight of about
200,000 or of four ring structures with molecular weights of about
800,000, as determined by electron microscopy. More recent
studies with enzyme prepared with the affinity column procedure
(33, D. R. Olsen, R. A. Berg, K. I. Kivirikko, and D. J. Prockop,
in preparation) have indicated that the ring structures were con-
taminants which were stable under the original staining conditions
for electron microscopy, whereas the enzyme dissociated partly
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Richard A. Berg and Darwin J. Prockop

J. Biol. Chem. 1973, 248:1175-1182.

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