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PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTOCOLLAGEN LYSINE HYDROXYLASE FROM CHICK EMBRYOS

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SUMMARY

A procedure was developed for partial purification of protocollagen lysine hydroxylase, the enzyme which synthesizes the hydroxylysine in collagen. The procedure involves fractionation with $(NH_4)_2SO_4$, fractionation with calcium phosphate gel, two successive chromatographies on DEAE-cellulose and gel filtration on 8% agarose. During the chromatography on DEAE-cellulose the protocollagen lysine hydroxylase was separated from protocollagen proline hydroxylase. The final preparation of the enzyme was up to 300-fold purified over the embryonic extract and up to 600-fold purified over the initial homogenate.

At least three different forms of the enzyme were observed. In low ionic strength buffers, the enzyme formed large, insoluble aggregates which were not studied further. Two major forms were obtained in high salt buffers, and these eluted from gel filtration columns with apparent molecular weights of about 550 000 and 200 000 as estimated from the elution positions of globular proteins. Some enzyme activity eluted between the two major forms, and this may have represented an additional form of the enzyme or an interconversion of the two forms during the chromatography.

The K_m values were determined for α-ketoglutarate, Fe^{2+} and ascorbate which are co-substrates or co-factors for the reaction and were found to be 50 μM, 1 μM and 50 μM, respectively. Even in the presence of saturating concentrations of these co-substrates and co-factors, maximal enzymic activity was not observed unless serum albumin, catalase and dithiothreitol were added to the incubation mixture. *p*-Mercuribenzoate inhibited the enzyme and the inhibition was partially reversed by dithiothreitol, indicating that free sulfhydryl groups were required for enzymic activity.

The results presented here demonstrate that protocollagen lysine hydroxylase is a separate enzyme from protocollagen proline hydroxylase but that it has several properties similar to those of the proline hydroxylase.

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INTRODUCTION

Essentially all the hydroxylysine in animal tissues is found in collagen. A number of studies have shown that the hydroxylysine in collagen is synthesized by the hydroxylation of lysine in a polypeptide precursor of collagen called protocollagen. The hydroxyproline in collagen is synthesized by the hydroxylation of proline in the same polypeptide precursor and protocollagen proline hydroxylase has now been extensively studied. The proline hydroxylase requires O_2, Fe^{2+}, α-ketoglutarate and a reducing agent which can be ascorbate (for review see ref. 1).

Initial studies indicated that the hydroxylation of lysine in protocollagen required the same co-factors or co-substrates as the hydroxylation of proline[2–5], and partially purified preparations of protocollagen proline hydroxylase were found to hydroxylate both proline and lysine in protocollagen[3]. Accordingly, it was originally suggested that both hydroxylations might be performed by the same enzyme[3]. Subsequently, however, highly purified preparations of protocollagen proline hydroxylase were shown not to contain any lysine hydroxylase activity[6] and experiments with competing polypeptides indicated that the enzymic site for the hydroxylation of lysine was separate from the enzymic site for the hydroxylation of proline[7].

In the present report we describe the separation of protocollagen lysine hydroxylase activity from protocollagen proline hydroxylase activity, and the partial purification and characterization of the lysine hydroxylase from chick embryos. During the preparation of this manuscript, MILLER[8] presented a preliminary report on the separation of the protocollagen lysine hydroxylase activity from the protocollagen proline hydroxylase activity of newborn rat skin.

MATERIALS AND METHODS

Lysine-labeled [^{14}C]protocollagen substrate

The lysine-labeled [^{14}C]protocollagen was prepared as described previously[3]. 72 cartilagenous tibiae from 12-day old embryos were incubated at 37° with 1 mM α,α'-dipyridyl in 2.5 ml of modified Krebs II medium containing glucose, phosphate buffer and inorganic salts[9]. 60 μC of [^{14}C]lysine, 250 μC/μmole (New England Nuclear Corp.), were added and the incubation was continued for 2.5 h. The tibiae were homogenized in 8 ml distilled water in a Teflon and glass homogenizer and centrifuged at 100 000 $\times$ g for 1 h. The supernate was dialyzed for 24 h against several changes of 20 mM Tris–HCl buffer adjusted to pH 7.8 at 4°. The sample was placed in boiling water for 10 min to destroy residual enzymic activity and it was stored frozen in aliquots of 200 000 disint./min.

A polypeptide which served as a substrate for the synthesis of hydroxylysine was synthesized by Mrs. K. Shudo, Dr. Y. Kishida and Dr. S. Sakakibara at the Peptide Center of the Institute for Protein Research, Osaka University, Osaka, Japan. The structure of the peptide was Ala–Arg–Gly–Ile–Lys–Gly–Ile–Arg–Gly–Phe–Ser–Gly, and it was prepared by coupling 4 separate tripeptide units with the general structure *tert.*-amyloxycarbonyl-X–Y–Gly. The details of the synthesis of the tripeptide units and of the coupling reactions will be described elsewhere (K. SHUDO, Y. KISHIDA and S. SAKAKIBARA, in preparation). The peptide was homogeneous by thin-layer chromatography, and by paper electrophoresis at pH 3.6 in 0.2 M acetate–

pyridine buffer, at pH 4.8 in 0.2 M acetate–pyridine buffer and in 0.2 M pyridine. The calculated elemental analysis for the peptide·4 CH_3COOH·3 H_2O was C 48.42, H 7.54, and N 17.59. The found values were C 48.33, H 7.55 and N 17.31 (ref. 10).

Assay procedures

In hydroxylations in which lysine-labeled [^{14}C]protocollagen was used as a substrate, 200 000 disint./min of the substrate were incubated in a final volume of 2 ml containing 0.05–0.2 units of enzyme, 0.05 mM $FeSO_4$, 0.5 mM α-ketoglutarate, 0.5 mM ascorbic acid, 0.1 mg/ml of catalase (Calbiochem), 0.1 mM dithiothreitol (Calbiochem), 1.5 mg/ml of bovine serum albumin (Sigma), and 50 mM Tris–HCl buffer adjusted to pH 7.8 at 25° (ref. 10). The samples were incubated at 37° for 30 min and the reaction was stopped by adding 20 ml of cold acetone[7]. After being allowed to stand in the cold for 30 min, the samples were centrifuged at about 3 000 × *g* for 30 min and the supernates were removed with suction. The pellets were dryed by gently blowing N_2 into the tubes and were suspended in 1 ml of distilled water followed by 6 ml of 0.3 M citrate–phosphate buffer, pH 6.4. Hydroxy[^{14}C]-lysine in the samples was assayed by periodate oxidation with a specific chemical procedure[11] using half the volume originally suggested.

In hydroxylations in which the synthetic polypeptide was used as substrate, 0.5 mg of the synthetic substrate was incubated in a final volume of 1.0 ml containing 0.5–2 units of enzyme, 0.05 mM $FeSO_4$, 0.1 mM α-[1-^{14}C]ketoglutarate (Calbiochem) which was diluted with non-labeled α-ketoglutarate (Calbiochem) in order to give the final specific activity of 35 000 disint./min per 0.1 μmole in initial experiments and 60 000 disint./min per 0.1 μmole in subsequent experiments, 0.5 mM ascorbic acid, 0.1 mg/ml catalase, Tris–HCl buffer adjusted to pH 7.8 at 25° (ref. 10). The polypeptide substrate was heated to 100° for 10 min and cooled to 0° just before addition to the incubation system. The samples were incubated at 37° for 40 min and the $^{14}CO_2$ was collected onto filter papers with a modification[10] of the assay described by RHOADS AND UDENFRIEND[12] for the assay of protocollagen proline hydroxylase. As reported elsewhere[10], quantitative amounts of hydroxylysine can be synthesized when the synthetic peptide is used as a substrate, and the amount of hydroxylysine synthesized is equimolar with the amount of $^{14}CO_2$ recovered in the assay.

The protein content of the enzyme preparations was measured by peptide absorbance at 225 nm using serum albumin as a standard, and by ninhydrin assays of acid hydrolysates.

Procedures for purification of the enzyme

Unless otherwise noted, all procedures were carried out at 0–4° and samples were stored at 0–4° without freezing between the various steps of the procedure. Also, all centrifugations were at 15 000 × *g*.

A total of 300–400 13-day old chick embryos were used as the source of the enzyme. Batches of 30 embryos each were homogenized in 0.2 M NaCl, 0.1 M glycine, 50 μM dithiothreitol, and 20 mM Tris–HCl buffer adjusted to pH 7.5 at 4° (1 ml of solution per g of embryo) in a Waring blender at full speed for 1 min. The homogenate was centrifuged for 30 min and solid $(NH_4)_2SO_4$ (Baker Chemical Company) was slowly stirred into the supernatant fraction to a final concentration of 17% saturation. The pellet obtained by centrifugation for 20 min was discarded and solid $(NH_4)_2SO_4$ was

slowly stirred into the supernatant fraction to a final concentration of 45% saturation. After centrifugation for 20 min, the pellet was dissolved in 0.15 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer adjusted to pH 7.4 at 4°. The sample in a final volume of about 1 500 ml was dialyzed for 3 h against 16 l of the same buffer and then for 16 h against an additional 16 l of the buffer. The sample was diluted to a volume of 2 700 ml and centrifuged for 20 min in order to remove a small amount of insoluble material.

The enzyme was precipitated with calcium phosphate gel by adding to the sample a suspension of the gel (Calbiochem) which had been diluted with distilled water to a concentration of 25 mg of solid per ml. The final amount of calcium phosphate gel added was generally 30 ml/g of protein, but the amount was varied from one-half to twice this amount depending upon the absorbing capacity of the particular batch of gel. After stirring the gel into the enzyme solution for 10 min, the gel was removed by centrifuging for 10 min. The pellet was then eluted step-wise with each of the following buffer solutions which were adjusted to pH 7.5 at 4°: (a) 0.04 M K_3PO_4, 0.1 M NaCl and 0.1 M glycine; (b) 0.06 M K_3PO_4, 0.15 M NaCl and 0.1 M glycine; (c), 0.09 M K_3PO_4, 0.15 M NaCl and 0.1 M glycine; (d) 0.15 M K_3PO_4, 0.15 M NaCl and 0.1 M glycine and (e) 0.18 M K_3PO_4, 0.15 M NaCl and 0.1 M glycine. In each of these elution steps the volume of buffer added was 2700 ml, the pellet was homogenized in the buffer with a Teflon and glass homogenizer, and then the sample was stirred with a magnetic stirrer in the buffer for 25 min. The eluates were removed by centrifuging for 10 min. The first eluate, (a), was discarded and eluates (b), (c), (d) and (e) were precipitated by adding solid $(NH_4)_2SO_4$ to a final concentration of 40% saturation. The precipitates were recovered by centrifuging the samples for 20 min and the pellets were then dissolved in 0.15 M NaCl, 0.1 M glycine, 50 μM, dithiothreitol and 20 mM Tris–HCl buffer, pH 7.5. The samples were dialyzed for 3 h and then overnight against 8 l of the same buffer. After the dialysis, the samples were centrifuged for 10 min to remove a small amount of insoluble material. Either one or two of the eluates of the calcium phosphate gel which contained the highest specific activity were combined and used for the DEAE-cellulose chromatography.

The DEAE-cellulose (DE 23, Whatman) column, 5 cm × 50 cm in size, was equilibrated with 0.15 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris-HCl buffer, pH 7.5 at 4°. The enzyme obtained from the calcium phosphate gel step was applied to the column at a flow rate of 300 ml/h, and the column was eluted at the same flow rate first with 100 ml of the equilibrating buffer followed by an 800-ml linear gradient from 0.15 to 0.25 M NaCl containing 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.5. The gradient was followed by 1200 ml of 0.25 M NaCl containing 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.5. The size of the fractions collected was 15 ml. Most of the enzyme activity did not absorb to the DEAE-cellulose under these conditions and the remaining activity was recovered within the first half of the gradient. The fractions containing more than 80% of the total enzyme recovered were pooled and concentrated by ultrafiltration through a Diaflo PM-30 membrane (Amicon) to a protein concentration of 8–10 mg/ml. The sample was centrifuged for 10 min to remove a small amount of insoluble material, and the clear supernatant was termed the DEAE-cellulose I enzyme pool. The first chromatography never gave more than 2- to 3-fold purification, but combined use of this step and a second DEAE-cellulose

chromatography in a column equilibrated with 0.07 M NaCl gave better results than either a single DEAE-cellulose chromatography, or two chromatographies in columns equilibrated with 0.07 M NaCl.

The DEAE-cellulose I enzyme pool was diluted with 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.5, to a final NaCl concentration of 0.07 M. The sample was then immediately applied to a DEAE-cellulose (DE 52, Whatman) column, 5 cm × 50 cm in size. The column was equilibrated with 0.07 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer pH 7.5, and eluted first with 300 ml of the same buffer followed by a 900-ml linear gradient of 0.07 to 0.15 M NaCl containing 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.5. The gradient was followed by 1000 ml of 0.15 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer pH 7.5. The size of the fractions was 15 ml. The enzyme was eluted as a sharp peak with about 0.12 M NaCl followed by a broad pattern of enzymic activity extending through the whole 0.15 M NaCl eluate and having a relatively low specific activity. Little additional enzymic activity could be eluted with higher NaCl concentrations. The sharp enzyme peak was pooled and concentrated to 8–10 ml by ultrafiltration through a Diaflo XM 100 A membrane (Amicon), and 0.40 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.4, was added to give a final NaCl concentration of 0.18–0.20 M. The sample was centrifuged for 10 min to remove a small amount of insoluble material, and the clear supernatant fluid was termed the DEAE-cellulose II enzyme pool.

The enzyme pool was applied to a 2.5 cm × 95 cm column of 8% agarose (Bio-Gel A-1.5 m 200–400 mesh, Bio-Rad) which was equilibrated and eluted with 0.2 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.4. The flow rate was 28 ml/h, and the size of the fractions 4.8 ml. The fractions containing most of the enzyme activity were pooled and termed the Bio-Gel A-1.5 m enzyme pool. This pool was usually concentrated by ultrafiltration to protein concentration of 3–5 mg/ml and stored at 0–4°.

RESULTS

Distribution of protocollagen lysine hydroxylase activity in homogenate fractions

Preliminary experiments indicated that protocollagen lysine hydroxylase was relatively insoluble in low ionic strength buffers. When the chick embryos were homogenized in the 0.01 M KCl and 20 mM Tris–HCl buffer used to extract protocollagen proline hydroxylase[13], only 50% of the activity appeared in the 15 000 × g supernate. The addition of 0.1% Triton X-100 did not significantly increase the amount of enzymic activity extracted in 0.01 M KCl and 20 mM Tris–HCl. When the chick embryos were homogenized in a buffer containing 0.1 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl, pH 7.5, about 80% of the activity was recovered in the 15 000 × g supernate and about 50% in the 150 000 × g supernate. When the chick embryos were homogenized in a buffer containing 0.20 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl, pH 7.5, approximately two-thirds of the enzymic activity was recovered in the 150 000 × g supernate (Table I). The results suggested that the enzyme readily formed aggregates in low ionic strength buffers.

TABLE I

DISTRIBUTION OF THE ACTIVITY OF PROTOCOLLAGEN LYSINE HYDROXYLASE IN CHICK EMBRYO HOMOGENATE FRACTIONS

Fifteen 13-day-old chick embryos (93 g wet weight) were homogenized as described in text. The homogenate was centrifuged at 15 000 × *g* for 30 min, and the 15 000 × *g* supernatant was centrifuged at 150 000 × *g* for 60 min. The two sediments were washed twice with the buffer used in the homogenization, and they were suspended in the same buffer before the assay. The results are expressed as disint./min of hydroxy [^{14}C]lysine synthesized in 30 min with 200 000 disint./min of [^{14}C]lysine-labeled protocollagen as substrate. An aliquot of each homogenate fraction corresponding to 10 mg wet weight of embryo was used as source for the enzyme.

Homogenate fraction	*Hydroxy [^{14}C]lysine synthesized (disint./min per 10 mg embryo)*	*Percent of total activity in homogenate*
15 000 × *g* sediment	610	12
150 000 × *g* sediment	1320	25
150 000 × *g* supernatant	3250	63

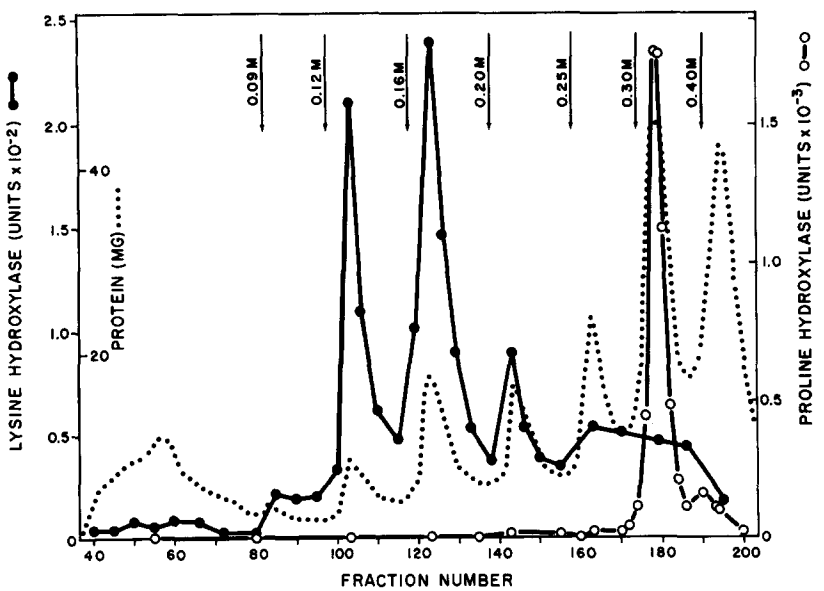

Fig. 1. Separation of protocollagen lysine hydroxylase from protocollagen proline hydroxylase by chromatography on DEAE-cellulose. In order to recover both enzymes the conditions for chromatography were different from those used in purifying the lysine hydroxylase. The sample was 3300 mg of the calcium phosphate gel enzyme pool. The column (DE-23, Whatman) was 5 cm × 44 cm and it was equilibrated with 0.07 M NaCl, 0.1 M glycine, 50 μM dithiothreitol and 20 mM Tris–HCl buffer, pH 7.4. Fractions of 15 ml were collected and the column was eluted by increasing the NaCl concentrations step-wise as indicated. The enzyme activities and protein are expressed as the amounts per 15 ml fraction. The protocollagen lysine hydroxylase eluted in several peaks in part because stepwise elution was used but a broad elution pattern was also seen when a linear gradient was used (see MATERIALS AND METHODS).

Separation of protocollagen lysine hydroxylase activity from protocollagen proline hydroxylase activity

The distribution of protocollagen lysine hydroxylase in homogenate fractions (Table I) was different from that previously observed with protocollagen proline hydroxylase, since with the latter enzyme over 95% of the enzymic activity was recovered in the 150 000 × *g* supernatant even when the buffer contained only 0.01 M salt[13]. Further differences between the enzymes were found in the $(NH_4)_2SO_4$ fractionation step and in the calcium phosphate gel step. Protocollagen lysine hydroxylase was precipitated with a lower concentration of $(NH_4)_2SO_4$ and a larger amount of calcium phosphate gel was required to absorb the enzyme. In spite of these differences the lysine hydroxylase obtained at the end of the calcium phosphate gel step still contained considerable amounts of protocollagen proline hydroxylase activity. However, a separation of the two enzymic activities could be obtained by a chromatography on DEAE-cellulose (Fig. 1). The two major peaks of protocollagen lysine hydroxylase were entirely free of any proline hydroxylase activity. The peak of protocollagen proline hydroxylase activity contained some lysine hydroxylase activity but as reported elsewhere[6] the proline hydroxylase is purified free of any lysine hydroxylase by a subsequent gel filtration step. The results indicated therefore that two separate enzymes are involved in the two hydroxylations.

Partial purification of protocollagen lysine hydroxylase

Five separate steps were used to purify the enzyme (Table II). Recovery of enzymic activity was high during $(NH_4)_2SO_4$ fractionation and there was about a two-fold purification during the step. The recovery of enzymic activity was 30–50% in the calcium phosphate gel step and there was generally about a 4-fold purification. The first chromatography on DEAE-cellulose is a crude procedure in which most of the lysine hydroxylase does not absorb to the column and the remaining activity is recovered in the first half of the gradient. However, since buffer containing 0.30 M NaCl is required to elute protocollagen proline hydroxylase (see Fig. 1), the enzyme

TABLE II

PARTIAL PURIFICATION OF PROTOCOLLAGEN LYSINE HYDROXYLASE

The starting material was the 15 000 × *g* supernatant of the homogenate of 1960 g wet weight of 13-day-old chick embryos. One unit of enzymic activity is defined as the amount of enzyme present in 1 mg of the $(NH_4)_2SO_4$ fraction (17–45% saturation) obtained from the 15 000 × *g* supernate of the chick embryo homogenate.

Enzyme fraction	*Total protein (mg)*	*Total units of enzyme*	*Specific activity*	*Purification**
15 000 × *g* supernatant	103 000	51 000	0.5	1
$(NH_4)_2SO_4$	47 600	47 600	1.0	2
Calcium phosphate gel	6 490	25 300	3.9	8
DEAE-cellulose I	2 170	18 400	8.5	17
DEAE-cellulose II	81.1	4 820	59.3	119
Bio-Gel A-1.5 m	23.5	2 900	123.4	247

* Purification calculated from specific activity of 15 000 × *g* supernatant fraction. When calculated on the basis of the specific activity of the embryonic homogenate, the degree of purification was about twice the values indicated.

recovered from the first DEAE-cellulose column is free of the proline hydroxylase. The second chromatography on DEAE-cellulose markedly improved the purification but there was a large decrease in total units of enzyme. Only the enzyme which eluted in an initial sharp peak (see MATERIALS AND METHODS) was pooled from this step. An additional 5000 units were recovered in a broad pattern on the descending side of the sharp enzyme peak, but these fractions had an average specific activity of only 14 and were therefore discarded. The remaining loss of activity was evidently explained by inactivation of the enzyme.

Six enzyme preparations were taken through the steps of the purification procedure. In four of the preparations the final enzyme was 160- to 250-fold purified over the original 15 000 $\times$ *g* supernatant, and the highest specific activity observed in a single fraction of the final gel filtration step was about 300 times that of the 15 000 $\times$ *g* supernatant. The purification calculated on the basis of specific activity of the original chick embryo homogenate is about twice as great or up to 600-fold.

Three enzyme preparations were subjected to a second agarose gel filtration. Although the elution pattern of protein suggested further purification of the enzyme, the loss of activity was large and as a result the specific activity decreased.

The overall recovery of the enzymic activity was 4–8% in the four best enzyme preparations. The relatively poor yields are largely explained by instability of the enzymic activity under all the experimental conditions tested so far, and it may be that the purification of enzyme protein was greater than that calculated on the basis of the specific activity. However, examination of the purified enzyme by sodium dodecyl sulfate polyacrylamide gel electrophoresis after treatment with mercaptoethanol and sodium dodecyl sulfate[14] indicated that it was not pure, since eight bands of polypeptide chains were seen.

Demonstration of multiple forms of protocollagen lysine hydroxylase

In the gel filtration used as the final step of the purification procedure the enzyme was recovered in at least two forms (Fig. 2). In three preparations a major part of the enzyme activity eluted from the 8% agarose column as a relatively broad peak with a maximum around Fraction 54, and a smaller amount eluted as a sharp peak around Fraction 64 (Fig. 2A). Some enzyme always eluted between the two major peaks and the descending limb of the Fraction 54 peak was always less sharp than the pattern obtained with standard proteins in the same column. In a fourth preparation the concentration of dithiothreitol was increased from 50 μM to 100 μM and in this preparation most of the enzyme was recovered as a peak in Fraction 64 of the gel filtration column (Fig. 2B). A smaller peak of enzyme was eluted in an intermediate position ahead of a Fraction 64 peak but it was retarded more than the first peak obtained with other preparations. However, with the higher concentration of dithiothreitol the enzyme lost activity rapidly. A similar loss of activity was observed when enzyme prepared with 50 μM dithiothreitol was dialyzed against 100 μM or 500 μM dithiothreitol.

Two of the six enzyme preparations were chromatographed on a 6% agarose (Bio-Gel A-5m) column instead of 8% agarose and the results are not directly comparable to those shown in Fig. 2.

Calibration of the agarose columns with standard proteins (Fig. 3) indicated that that if both forms of the enzyme were assumed to be globular proteins, the larger

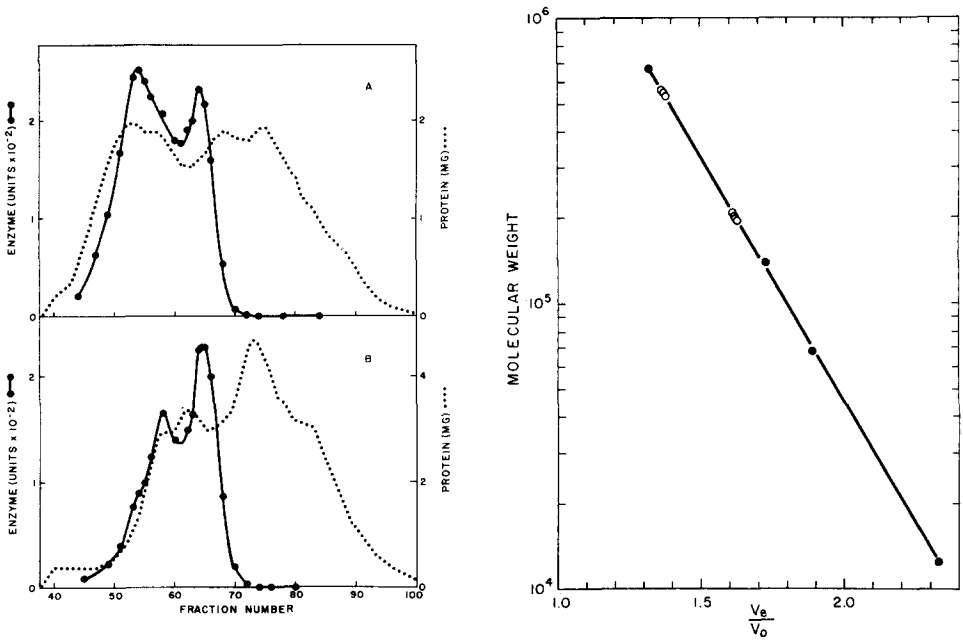

Fig. 2. A. Gel filtration in the last step of the purification of one preparation of protocollagen lysine hydroxylase. The 8% agarose column (A-1.5 m, Bio-Rad) was 2.5 cm × 95 cm and fractions of 4.8 ml were collected. Other conditions were as described in MATERIALS AND METHODS. Two other enzyme preparations gave similar patterns in this step (see text). B. Gel filtration in the last step of the purification of one preparation of protocollagen lysine hydroxylase. Conditions were the same as described above.

Fig. 3. Calibration of the gel filtration column shown in Figs. 2A and 2B with standard proteins. The protein standards used were bovine thyroglobulin (Sigma), ceruloplasmin (Sigma), bovine serum albumin (Sigma), and cytochrome *c* (Sigma). Symbols: elution positions of the protein standards (●); elution positions of the larger form of enzyme obtained with three enzyme preparations (Fig. 2A) and the smaller form obtained with four enzyme preparations (Figs. 2A and 2B) (○).

form had a molecular weight of about 550 000 and the smaller form had a molecular weight of about 200 000. The enzyme eluted between the two forms may have represented a form of the enzyme with an intermediate molecular weight or it may be explained by interconversion of the two major forms during the chromatography. Of interest was the fact that none of the experiments revealed an active form of the enzyme with an apparent molecular weight less than 200 000, and in all the gel filtration chromatograms the descending limb of the peak of the smaller form of the enzyme was relatively sharp.

Other properties of the partially purified enzyme

As reported previously[2–5], the synthesis of hydroxylysine by protocollagen lysine hydroxylase required as co-substrates or co-factors α-ketoglutarate, Fe^{2+}, O_2 and a reducing agent such as ascorbate. Using the synthetic polypeptide substrate for the synthesis of hydroxylysine and the α-[^{14}C]ketoglutarate assay, it was possible to determine the K_m value for Fe^{2+}, α-ketoglutarate and ascorbate for the first time

(Table III). The K_m for Fe^{2+} was 1 μM or about the same as the value of 1–5 μM observed with protocollagen proline hydroxylase[3,15,16]. The K_m for α-ketoglutarate was 50 μM or somewhat higher than the value of 5–10 μM which was observed with protocollagen proline hydroxylase[3,15,16]. The K_m for ascorbate was 50 μM, a value which is somewhat less than the value of 100–400 μM observed with protocollagen proline hydroxylase[3,15,16].

TABLE III

K_m VALUES FOR Fe^{2+}, α-KETOGLUTARATE AND ASCORBATE IN HYDROXYLATION OF THE SYNTHETIC PEPTIDE BY PROTOCOLLAGEN LYSINE HYDROXYLASE

The enzymic reaction was carried out as described in MATERIALS AND METHODS using the synthetic polypeptide as the substrate. The concentration of the co-factor or co-substrate under study was varied over a 100-fold range and the K_m was obtained from double reciprocal plots of $^{14}CO_2$ and the co-factor or co-substrate concentration.

Co-factor or co-substrate	*K_m value (M)*
Fe^{2+}	$1 \cdot 10^{-6}$
α-Ketoglutarate	$5 \cdot 10^{-5}$
Ascorbate	$5 \cdot 10^{-5}$

The concentrations of all these cofactors in the standard conditions for assaying the lysine hydroxylase were at least 10 times the K_m concentrations except that when the α-[^{14}C]ketoglutarate assay was used, the α-ketoglutarate concentration was reduced to only twice the K_m. Higher concentrations of α-[1-^{14}C]ketoglutarate produced higher blank values of $^{14}CO_2$ and higher concentrations of unlabeled α-ketoglutarate reduced the sensitivity of the assay[10].

As reported elsewhere[10], the K_m for the synthetic substrate used to assay the protocollagen lysine hydroxylase was 0.5 mg/ml or 0.4 mM. Because only limited amounts of the synthetic substrate were available, the concentration employed under standard conditions for the assay was the K_m concentration. Using this concentration of substrate did not affect the assay of enzymic activity, probably because less than 2% of the substrate was hydroxylated under the standard assay conditions.

The amount of hydroxylysine synthesized by the partially purified enzyme was calculated on the basis of the specific activity of the α-[^{14}C]ketoglutarate[10]. The results indicated that 1 mg of the partially purified enzyme synthesized approximately 0.6 μmole of hydroxylysine in 1 h at 37° when the hydroxylation was carried out with saturating concentrations of the synthetic substrate and all co-factors except α-ketoglutarate. To date, however, it has not been possible to compare the v_{max} and K_m of the synthetic substrate to that of the natural substrate, protocollagen. Therefore it is not known whether the enzyme synthesizes hydroxylysine at a more rapid rate with the natural substrate.

The pH optimum of the hydroxylating reaction was about 7.4 (Fig. 4). Because the pH of Tris–HCl buffer decreases about 0.3 units when the temperature is raised from 25° to 37°, the buffer used to assay the enzyme was adjusted to pH 7.8 at 25°.

As reported previously[15–17], the activity of protocollagen proline hydroxylase is increased by the addition of bovine serum albumin, catalase and dithiothreitol to the enzymic system. Similar effects were observed here with protocollagen lysine hydroxylase (Table IV). When all three agents were omitted, the activity was reduced

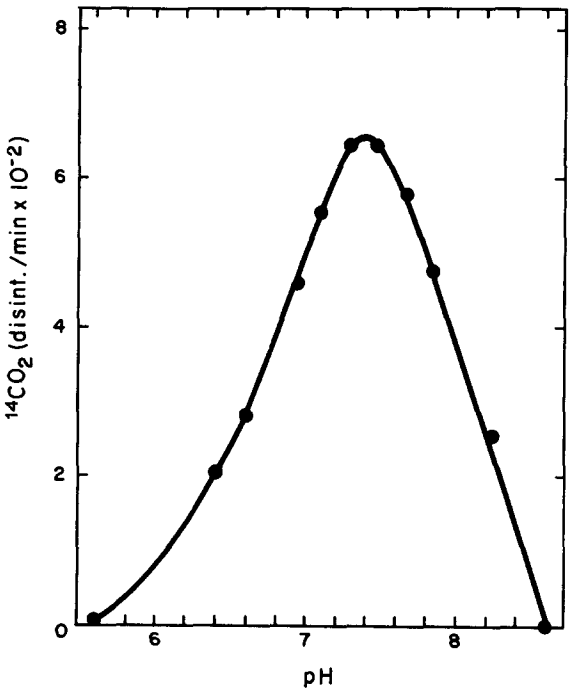

Fig. 4. pH optimum of protocollagen lysine hydroyxlase. Conditions were as described in MATERIALS AND METHODS. The enzyme was assayed by the evolution of $^{14}CO_2$ from α-[1-^{14}C]ketoglutarate.

TABLE IV

EFFECT OF BOVINE SERUM ALBUMIN, DITHIOTHREITOL, AND CATALASE ON THE SYNTHESIS OF HYDROXYLYSINE BY PROTOCOLLAGEN LYSINE HYDROXYLASE

Protocollagen lysine hydroxylase was incubated with 200 000 disint./min of [^{14}C]lysine-labeled protocollagen as described in text.

Expt. No.	*Additions or omissions*			*Hydroxy [^{14}C]lysine synthesized (disint./min)*	*Percent of control mean*
	Bovine serum albumin	*Dithiothreitol*	*Catalase*		
1	+	+	+	6960	
	+	+	+	7360	
	+	+	+	7500	
	−	−	−	2800	39
	−	−	+	3990	55
	−	+	−	4350	60
	+	−	−	5390	74
2	+	+	+	5850	
	+	+	+	6650	
	+	+	+	6920	
	−	+	+	4040	62
	+	−	+	5870	91
	+	+	−	5160	80

to 39% of the control value and addition of any single agent increased the activity (Expt. 1, Table IV). However, no single agent fully restored the activity and addition of two of the agents did not give maximal activity. Although the single omission of dithiothreitol gave a value which was only slightly below the control mean, further experiments with the synthetic substrate (not shown) demonstrated that this was a consistent finding. The concentrations of bovine serum albumin, catalase and dithiothreitol used in this experiment and under the standard conditions for assaying the enzyme were found to be optimal.

Protocollagen proline hydroxylase was previously shown to be inhibited by *p*-mercuribenzoate[6,18]. Again a similar effect was observed here with protocollagen

TABLE V

EFFECT OF *p*-MERCURIBENZOATE ON THE ACTIVITY OF PROTOCOLLAGEN LYSINE HYDROXYLASE

Protocollagen lysine hydroxylase, 14 μg, was preincubated for 15 min at 37° in 1.0 ml of 20 mM Tris–HCl buffer adjusted to pH 7.8 at 25°. *p*-Mercuribenzoate was present during preincubation in the concentrations indicated. After preincubation, 0.5 ml of the sample was transferred into tubes containing 200 000 disint./min of [^{14}C]lysine-labeled protocollagen substrate, and the co-factors and co-substrates in a final volume of 2.0 ml. The incubation was continued for 30 min with *p*-mercuribenzoate in the concentrations indicated. The concentrations of co-factors or co-substrates were the same as in standard hydroxylations, except that dithiothreitol and bovine serum albumin were not used.

Addition	*Hydroxy [^{14}C]lysine synthesized (disint./min)*	*Inhibition (%)*
None	1850	
	2150	
	2240	
1 μM *p*-mercuribenzoate	830	60
10 μM *p*-mercuribenzoate	110	95
100 μM *p*-mercuribenzoate	<100	>95
100 μM *p*-mercuribenzoate *plus* 200 μM dithiothreitol*	890	57
	910	56
200 μM dithiothreitol*	2880	(−10)

* Dithiothreitol was added after preincubation for 10 min.

lysine hydroxylase (Table V). With as little as 1 μM *p*-mercuribenzoate the enzyme was inhibited over 50%. However, the inhibition observed with even 100 μM *p*-mercuribenzoate was partly reversed by the addition of 200 μM dithiothreitol (Table V). These results suggested that free sulfhydryl groups are required for activity of protocollagen lysine hydroxylase.

DISCUSSION

The synthesis of hydroxyproline by protocollagen proline hydroxylase has been extensively studied during recent years[1] but there is little information about the synthesis of hydroxylysine in collagen. In fact, the data presented here and those recently reported in preliminary form by MILLER[8] are the first clear demonstration that a separate enzyme is required for the hydroxylation of lysine in protocollagen. Differences between the lysine hydroxylase and the proline hydroxylase were observed

in the solubility in low ionic strength buffers, precipitation by ammonium sulfate and absorption to calcium phosphate gel. Chromatography on DEAE-cellulose provided a complete separation of protocollagen lysine hydroxylase from protocollagen proline hydroxylase.

Previous attempts to purify protocollagen lysine hydroxylase have been hampered by the lack of suitable substrates and convenient assays for the enzyme. These difficulties have been overcome by the recent development of a rapid assay based on the stoichiometric release of $^{14}CO_2$ from α-[1-^{14}C]ketoglutarate during the hydroxylation of lysine, and the synthesis of peptide substrates with amino acid sequences comparable to those around glycosylated hydroxylysine in collagen[10].

With the procedures developed here the protocollagen lysine hydroxylase was purified up to 300-fold based on the specific activity of the extract of chick embryos or up to 600-fold based on the specific activity of the initial homogenate. The recovery of activity was relatively low and complete purification was not achieved, in part because of the instability of the enzyme and in part because it assumed several different forms. At least three different forms were observed. In low ionic strength buffers, the enzyme formed large, insoluble aggregates which were not studied further. The two major forms which were partially purified here eluted from gel filtration columns with apparent molecular weights of about 550 000 and 200 000. Some enzyme activity eluted between the major forms, and this may have reflected the presence of still another form of the enzyme, or interconversion of the two main forms during the chromatography. The form with an apparent molecular weight of 200 000 was the smallest active form recovered in any of the enzyme preparations. Because the shape of the molecule is not known, it is not clear whether the form with an apparent molecular weight of 550 000 is a dimer, trimer or tetramer of the smaller form.

As reported earlier[2-5], the synthesis of hydroxylysine by the enzyme required as co-factors or co-substrates α-ketoglutarate, Fe^{2+}, and a reducing agent such as ascorbate. The K_m values for these co-factors or co-substrates were measured for the first time and they were found to be only slightly different than the K_m values for the same co-factors or co-substrates for protocollagen proline hydroxylase. Even in the presence of saturating concentrations of these co-factors and co-substrates, maximal activity was not observed unless bovine serum albumin, catalase and dithiothreitol were added to the incubation mixture. Similar phenomena have previously been observed with protocollagen proline hydroxylase[15-17] but the mechanism of the effects has not been defined for either enzyme. *p*-Mercuribenzoate inhibited the enzymic activity and dithiothreitol partially reversed the inhibition, indicating that free sulfhydryl groups are required for enzymic activity. Again these results are similar to previous observations with protocollagen proline hydroxylase[6,18].

The results presented here indicate that protocollagen lysine hydroxylase and protocollagen proline hydroxylase are separate enzymes but that they have a number of similar properties.

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