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PARTIAL PURIFICATION AND PROPERTIES OF COLLAGEN LYSINE HYDROXYLASE FROM CHICK EMBRYOS*

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SUMMARY

The enzyme collagen lysine hydroxylase, which converts some of the lysine residues in a newly synthesized collagen chain to 5-hydroxylysine residues, has been partially purified from extracts of chick embryo tissues by ammonium sulfate fractionation, calcium phosphate gel treatment, and stepwise elution from DEAE-cellulose. The enzyme so prepared resembles collagen proline hydroxylase in that its activity is increased by bovine serum albumin and it is protected from inactivation by glycine. It differs from the latter enzyme in that it has a higher pH optimum. The most purified preparations are completely devoid of proline hydroxylating activity, which confirms previous assumptions that two separate enzymes are responsible for hydroxylating the two amino acids in collagen.

INTRODUCTION

The formation of hydroxylysine from lysine after the latter is incorporated into a collagen precursor polypeptide chain is an enzymatic process. Like the hydroxylation of proline, lysine hydroxylation has been shown to proceed by a direct introduction of the hydroxyl group onto C-5 of lysine[1] and to require as cofactors and co-substrates molecular oxygen[2], Fe^{2+}, and α-ketoglutaric acid[3,4]. There is also a less specific requirement for ascorbic acid[3,4]. It was assumed at one time that a single enzyme carried out the hydroxylation of both proline and lysine, but recent experiments have made it seem more probable that two separate enzymes are involved: certain polyamino acids which inhibit proline hydroxylation in a collagen precursor polypeptide chain have no effect on lysine hydroxylation[5], and a highly purified collagen proline hydroxylase had almost no ability to bring about the hydroxylation of lysine residues[6].

A procedure for the partial purification of collagen lysine hydroxylase in a form completely free from collagen proline hydroxylase is described below, and some properties of the enzyme are reported.

* This paper is dedicated to the memory of our colleague, Donald D. Van Slyke, whose collaboration and friendship we enjoyed for many years.

MATERIALS AND METHODS

Materials

All chemicals used were of Analytical Reagent Grade or of the highest purity obtainable. Special reagents were obtained from the following sources: L-[^{14}C]lysine, uniformly labeled, specific activity 210 mC/mmole, from International Chemical and Nuclear Corp.; "Aquasol" proprietary scintillation counting fluid from New England Nuclear Corp.; Tris and $(NH_4)_2SO_4$ (Special Enzyme Grade) from Schwarz/Mann. DEAE-cellulose was Whatman DE-52, and agarose was Sepharose 6B from Pharmacia. Calcium phosphate gel was either purchased from Calbiochem or prepared in the laboratory by the method of KEILIN AND HARTREE[7]; the two products gave essentially the same results.

Protein was determined by the Folin–Lowry method[8]. Except where indicated, the pH of all buffers was determined at room temperature.

Assay of lysine hydroxylase activity

The assay mixture contained the following substances: α-ketoglutaric acid, 0.5 mM; Tris–HCl buffer, pH 8.0 (measured at 25°), 62.5 mM; ascorbic acid, 3 mM; $FeSO_4$, 0.125 mM; catalase, 0.05 mg/ml; substrate labeled with [^{14}C]lysine, 80 000 to 100 000 counts/min, approximately 0.3 mg protein; enzyme solution as appropriate. When assaying samples from the DEAE-cellulose fractionation, bovine serum albumin, 1 mg/ml, was also added. The total volume was 2 ml. After incubating the mixture in air at 30° for 1.5 h (except where otherwise indicated), the reaction was stopped by the addition of 2 ml conc. HCl, and the tubes were sealed and heated at 110° for 18 to 22 h. Lysine and hydroxylysine were separated on the Beckman–Spinco amino acid analyzer with a 9.5-cm column of resin PA-35 at 55° and the 0.38 equiv/l, pH 4.26 buffer. Appropriate 2-ml fractions of the effluent were collected, mixed with 10 ml of "Aquasol", and counted in a scintillation counter at 90% efficiency.

The substrate, prepared essentially according to HUTTON *et al.*[9], was [^{14}C]lysine, incorporated into a collagen-like protein in the presence of α,α'-dipyridyl to inhibit hydroxylation. Typically, 500 μC [^{14}C]lysine was incubated with 18 g minced tissues from 9-day-old chick embryos, and the protocollagen substrate was subsequently extracted into 36 ml of 0.5 M acetic acid.

Purification of enzyme

Collagen lysine hydroxylase was prepares from chick embryos. All procedures were carried out at 0 to 4°. Homogenization and extraction of the embryos and the first $(NH_4)_2SO_4$ fractionation (15 to 40% precipitate) were carried out exactly as described[10] for the purification of collagen proline hydroxylase. From this point on, unless otherwise indicated, all buffers contained 0.1 M glycine and 0.1 mM dithiothreitol. The $(NH_4)_2SO_4$ precipitate was resuspended in 0.05 M Tris buffer, pH 7.6, and dialyzed exhaustively against the same buffer. After removal of some insoluble material by centrifugation (18 000 $\times$ *g*, 20 min), the protein concentration was adjusted to 15 mg/ml by dilution with buffer.

Calcium phosphate gel fractionation

This step was done by the procedure of HALME *et al.*[6], modified slightly. Calcium

phosphate gel suspension (12 mg/ml) was added in four aliquots, the first consisting of 2.5 ml per g protein and the rest of 10 ml per g protein in the original solution. (Collagen proline hydroxylase is largely removed with the second, third and fourth additions and can be treated separately.) After removal of the final aliquot of calcium phosphate gel, protein in solution was recovered by $(NH_4)_2SO_4$ precipitation (60% of saturation) and dialyzed thoroughly against the starting buffer for the DEAE-cellulose fractionation. Before the resulting solution was applied to the DEAE-cellulose column, it was clarified by centrifugation for 15 min at 105 000 $\times$ g in the Spinco preparative ultracentrifuge.

DEAE-cellulose fractionation

Typically, for 2.2 g of protein from the preceding step in a volume of 300 ml, a column of DEAE-cellulose 2.5 cm $\times$ 80 cm was equilibrated with 0.05 M Tris buffer, pH 7.6. After the sample had been pumped in, the column was washed with the equilibrating buffer until the absorbance at 280 nm had returned to the base line. The column was eluted in a stepwise fashion with the same buffer containing 0.03 M, 0.06 M, 0.08 M, and 0.10 M KCl. Collagen lysine hydroxylase usually appeared in the 0.08 M and 0.10 M KCl fractions.

RESULTS

Assay for collagen lysine hydroxylase

Although it is well established that the co-factor requirements for collagen lysine hydroxylase are the same as for collagen proline hydroxylase, the conditions we have previously used in assaying proline hydroxylase[10] had to be modified for the present work. Increasing the ascorbic acid concentration from 1.05 mM (used in the proline hydroxylase assay) to 2 to 4 mM resulted in a 90% increase in the amount of hydroxylysine produced by crude enzyme preparations, although the amount of hydroxylysine produced by more purified preparations was not enhanced by such an increase. The rate of proline hydroxylation is almost maximal in the presence of 0.05 mM α-ketoglutarate, but the rate of hydroxylysine production was increased about 25% by raising this concentration to 0.5 mM.

RHOADS *et al.*[11] showed that the addition of bovine serum albumin and dithiothreitol to purified preparations of collagen proline hydroxylase greatly stimulated the apparent activity, but the effect was less in less purified preparations and in assay systems with larger concentrations of enzyme protein[12]. We have found that addition of bovine serum albumin to the assay mixture can stimulate collagen lysine hydroxylation as well. For example, in assaying a fraction from the DEAE-cellulose step, the addition of 1 mg albumin per ml resulted in a 2-fold stimulation when the enzyme protein concentration was 16 μg/ml, and a 4-fold stimulation when it was 8 μg/ml. Possibly additional albumin would have produced further stimulation, but we have avoided adding more than 1 mg albumin per ml in order to keep down the total amount of protein hydrolysate applied to the ion-exchange column. For the same reason, no albumin was added to the assay mixture at any stage prior to the DEAE-cellulose step.

The enzyme exhibited a pH optimum between pH 8.0 and 8.4 (Fig. 1). This is significantly higher than the pH optimum of 7.3 to 7.4 found by KIVIRIKKO AND

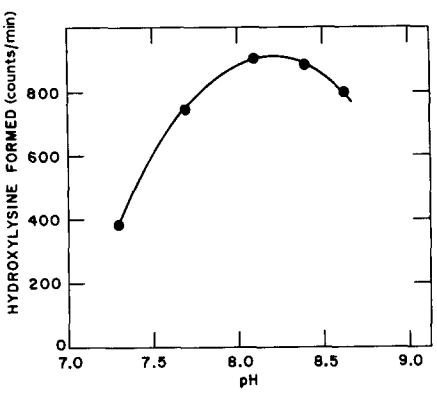

Fig. 1. Dependence of collagen lysine hydroxylase activity on pH. Assays were carried out as described under MATERIALS AND METHODS except that Tris buffers of varying pH were used. The pH was determined at 30° in samples devoid of ^{14}C-labeled substrate but otherwise duplicates of the assay samples.

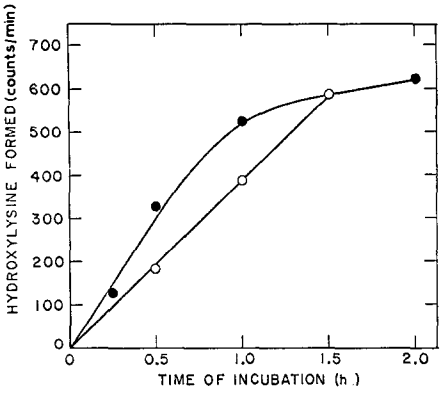

Fig. 2. Time course of the hydroxylation of lysine residues in protocollagen. Assays were carried out as described in the text except that the reaction was terminated at various times and was carried out either at 30° (○—○) or at 37° (●—●).

PROCKOP[13] for collagen proline hydroxylase with the synthetic substrate $(Pro\text{–}Gly\text{–}Pro)_n$.

Fig. 2 shows the time course of the hydroxylation at 30° and at 37°. At 37° the rate of reaction is approximately constant for the first hour and then drops significantly. At 30° the rate is constant for the first 1.5 h. We chose, for convenience, to use a 30° incubation for 1.5 h, but it would be almost as satisfactory to incubate for 1 h at 37°.

Purification of collagen lysine hydroxylase

The results of a typical purification are shown in Table I.

TABLE I

PURIFICATION OF COLLAGEN LYSINE HYDROXYLASE FROM CHICK EMBRYOS

Details of the purification are given in the text. Embryo tissues were homogenized (Virtis homogenizer) with an equal weight of 0.01 M KCl and centrifuged (35 000 × *g*, 20 min). The sediment was re-homogenized with 0.47 of the previous volume of 0.01 M KCl and centrifuged. The combined supernatant solutions constituted the original tissue extract. Typically, it contained 31.2 g of protein extracted from 955 g of embryo tissue.

Step	*Recovery*		*Purification factor*
	% of starting protein	*% of starting enzyme activity*	
Original tissue extract	100	100	
15–40% $(NH_4)_2SO_4$	36	40	1.08
Calcium phosphate gel	10	40	4
DEAE-cellulose:			
0.06 M KCl eluate	0.9	13	14
0.08 M KCl eluate	0.3	6	20
0.10 M KCl eluate	0.5	8	16

Factors which stabilize collagen lysine hydroxylase

Glycine, which was shown by HALME AND KIVIRIKKO[14] to protect collagen proline hydroxylase against inactivation in some as yet unexplained way, also affords some protection to collagen lysine hydroxylase. For example, an enzyme solution (combined 0.08 M and 0.10 M KCl fraction from the DEAE-cellulose step), dialyzed for 48 h at 2° against 0.05 M Tris buffer, pH 7.6, containing 0.1 M glycine, lost no significant amount of activity, but, if the glycine was omitted, the solution lost 20% of its enzymatic activity. Although no extensive study if the limits of this protection has been made, glycine at a concentration of 0.1 M was incorporated into all buffers used in this work.

Dithiothreitol (0.1 mM) in some similar experiments appeared to afford slight protection to collagen lysine hydroxylase activity, and in others it had no effect. Since we have seen no indication that at this concentration dithiothreitol destroys enzyme activity, it was also added to all buffers.

Molecular size of collagen lysine hydroxylase

When the product from the DEAE-cellulose step was concentrated and subjected to gel filtration on 6% agarose (Sepharose 6B, 1.8 cm × 150 cm) in 0.05 M Tris buffer, pH 7.6, 0.1 M glycine, 0.1 M KCl, the peak of enzyme activity was found in the same fraction as the peak of collagen proline hydroxylase when run on the same column. Thus, the two hydroxylases probably are similar in size under these conditions. HALME *et al.*[6] found, by gel filtration, that chick embryo collagen proline hydroxylase exhibited a molecular weight of about 350 000, and experiments in our own laboratory (E. A. POPENOE AND R. B. ARONSON, unpublished) confirmed this value. At the present time the correct value for the molecular weight of proline hydroxylase in its native state is uncertain, however[15,16], so that any conclusion as to the true molecular weight of lysine hydroxylase must await further experiments with highly purified enzyme.

TABLE II

SEPARATION OF COLLAGEN LYSINE HYDROXYLASE AND COLLAGEN PROLINE HYDROXYLASE

The enzyme preparations were obtained from the purification scheme described in the text. The calcium phosphate fraction was the unadsorbed material remaining after the final aliquot of calcium phosphate gel had been removed. The DEAE-cellulose fraction was the 0.08 M KCl eluate. Lysine hydroxylase activity was determined as described in the text. Proline hydroxylase activity was determined by the method of HUTTON *et al.*[9], as previously described[10]. This assay method is based on determination of the amount of ^3H released into the water of the medium during hydroxylation of protocollagen labeled with L-[3,4-^3H]proline.

Enzyme preparation	*Hydroxylase assayed*	*Enzyme protein concentration in assay mixture (μg/ml)*	*Hydroxylysine formed (counts/min)*	*^3H_2O formed counts/min*
$(NH_4)_2SO_4$	Lysine	120	300	—
	Proline	19	—	370
Calcium phosphate gel	Lysine	58	495	—
	Proline	18	—	130
DEAE-cellulose	Lysine	800	3000	—
	Proline	1600	—	15

Separation of the enzyme hydroxylating lysine and proline in collagen

Enzyme assays on preparations from various steps in the purification (Table II) show that the calcium phosphate gel step affords a major separation between the two hydroxylase activities. All proline hydroxylase remaining with the lysine hydroxylase at this stage is completely removed in the DEAE-cellulose step. This result was to be expected since, under similar conditions, proline hydroxylase is eluted from the column at KCl concentrations between 0.2 and 0.4 M (ref. 10) whereas lysine hydroxylase is eluted at concentrations between 0.06 and 0.10 M.

DISCUSSION

It is not surprising to find that collagen proline hydroxylase and collagen lysine hydroxylase have much in common in view of the similarity in the reactions they catalyze. Lysine hydroxylase is stimulated by the presence of albumin in the reaction mixture and protected against inactivation by the presence of glycine in all solutions. Both these characteristics had been previously found for proline hydroxylase[11,14].

It appears that higher concentrations of α-ketoglutarate are required to saturate lysine hydroxylase than are needed to saturate proline hydroxylase. The variability of the requirement for ascorbate may be caused by enzymatic destruction of ascorbate in the crude preparations used in the early stages of this work, or may reflect the fact that it does not participate directly in the hydroxylation reaction. In studying the co-factor and substrate requirements of collagen proline hydroxylase, HUTTON *et al.*[17] found that *in vitro* hydroxylation could proceed in the presence of either ascorbate or any one of several other reducing agents. WEINSTEIN *et al.*[5] reported that lysine hydroxylation also could proceed in the presence of reducing agents other than ascorbate.

The results presented in Table II demonstrate clearly that two separate enzymes are responsible for the hydroxylation of proline and lysine residues in collagen. WEINSTEIN *et al.*[5] had previously demonstrated that hydroxylation of proline and lysine in protocollagen involves two separate enzymatic sites, since polypeptides which inhibited hydroxylation of prolyl residues (*e.g.* polyproline) do not affect hydroxylation of lysyl residues. Furthermore, HALME *et al.*[6] reported that highly purified collagen proline hydroxylase did not synthesize significant amounts of hydroxylysine from [^{14}C]lysine. However, as WEINSTEIN *et al.*[5] pointed out, the procedures used to purify the proline hydroxylase may have selectively destroyed lysine-hydroxylating activity in the same protein. The isolation of a lysine-hydroxylating enzyme preparation which is devoid of proline-hydroxylating activity, reported in the present work, proves that there are two separate enzymes, as does the demonstration that the two activities are eluted from DEAE-cellulose under different conditions.

MILLER[18] has recently reported obtianing similar results with preparations from newborn rat skins.

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