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ISOLATION AND PARTIAL CHARACTERIZATION OF HIGHLY PURIFIED PROTOCOLLAGEN PROLINE HYDROXYLASE*

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SUMMARY

A procedure for the isolation of relatively stable preparations of highly purified protocollagen proline hydroxylase is reported. The most purified enzyme preparation was homogeneous in the analytical ultracentrifuge, and showed only one minor contaminant in disc electrophoresis. This enzyme preparation synthesized about 600 μg of hydroxyproline per mg protein per h under the assay conditions used, with a saturating concentration of the synthetic polytripeptide $(Pro–Gly–Pro)_n$, molecular weight 6600, as substrate.

The enzyme had an $s_{20,w}$ of 6.7. However, the elution pattern in gel filtration suggested a molecular weight of about 350 000. Amino acid analysis indicated that the enzyme preparation contained relatively large amounts of aspartic acid, glutamic acid, serine, glycine and lysine.

The activity of the purified enzyme was strongly inhibited by *p*-mercuribenzoate, and the inhibition could be reversed by dithiothreitol. *N*-Ethylmaleimide was a less effective inhibitor. The results suggest that free sulphydryl groups are required for the activity of the enzyme.

Although partially purified preparations of the enzyme hydroxylate lysine as well as proline residues in protocollagen, the highly purified enzyme preparations did not hydroxylate lysine residues. Thus two separate enzymes, protocollagen proline hydroxylase and protocollagen lysine hydroxylase, are probably involved in the hydroxylation of proline and lysine in protocollagen.

INTRODUCTION

The enzyme protocollagen proline hydroxylase synthesizes the hydroxyproline in collagen by the hydroxylation of proline which has been incorporated into proto-

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collagen, the proline-rich[1–5] and lysine-rich[6] polypeptide precursor of collagen. The enzyme does not hydroxylate free proline, proline in tripeptides or proline in poly-L-proline[7], but it catalyses the hydroxylation of proline in synthetic polytripeptides with the structure $(Gly–X–Pro)_n$ in which "X" is proline[7–12] or alanine[13–14] but not glycine[14], and in denatured cuticle collagen from *Ascaris lumbricoides*[15]. Poly-L-proline, Form II[16–18], and $(Gly–Pro–Gly)_n$ (ref. 14) are competitive inhibitors of the enzyme.

Protocollagen proline hydroxylase has previously been partially purified from chick embryo extracts[7,16,19], and from the skin of newborn rats[20]. The synthesis of hydroxyproline by these partially purified preparations of the enzyme has been shown to require O_2, Fe^{2+}, α-ketoglutarate and ascorbate (see refs. 7 and 19). The same cofactors or cosubstrates are required for the hydroxylation of lysine in protocollagen[6,7,16,21,22], and preparations of protocollagen proline hydroxylase which have been purified several hundred-fold from chick embryo extracts have been found to hydroxylate lysine as well as proline residues in protocollagen[16].

The most purified preparations of protocollagen proline hydroxylase obtained from chick embryo extracts with previous procedures[16] have rapidly lost their activity. It has therefore been difficult to work with these preparations, and previous attempts at further purification of the enzyme have been unsuccessful. A recent study[23] indicated, however, that protocollagen proline hydroxylase is relatively stable in buffers containing free glycine and having a pH around 8. This has enabled us to produce highly purified and relatively stable preparations of protocollagen proline hydroxylase.

MATERIALS AND METHODS

Materials

The polytripeptide with the structure $(L\text{-Pro–Gly–L-Pro})_n$ was purchased from Miles-Yeda, Kiryat Weizmann, Rehovoth[9,24]. The polymer fractions used in the present study had average molecular weights of 6600 and 8000. The two preparations had the same K_m and v_{max} values when used as substrates for protocollagen proline hydroxylase[9]. Lysine-labelled and proline-labelled [^{14}C]protocollagen were prepared from tibiae of 10-day-old chick embryos as described previously[7,16]. After the last dialysis the preparations were boiled[25] for 5 min before the former was divided for storage into aliquots of 300 000 disint./min containing about 2 μg lysine-labelled protocollagen, and the latter of 50 000 distint./min containing about 0.3 μg proline-labelled protocollagen. Uniformly labelled L-[^{14}C]lysine (220 μC/μmole) and L-[^{14}C]-proline (180 μC/μmole) were purchased from New England Nuclear Corp., Boston, Mass.; Bio-Gel A-1.5 m, 100–200 mesh, containing 8% agarose from Bio-Rad Laboratories, Richmond, Calif.; and DEAE-cellulose, NM 2100 DEAE, from Macherey, Nagel, Düren. IgM globulin was obtained from a patient with macroglobulinaemia.

Purification of protocollagen proline hydroxylase

A total of 200 13-day-old chick embryos was used as the source of enzyme. The $(NH_4)_2SO_4$ fractionation (30–65% satn.) of the embryo extract was prepared as described previously[9], and the dissolved precipitate was dialysed overnight against

0.1 M NaCl, 0.1 M glycine, and 0.01 M Tris–HCl buffer (pH 7.8). The calcium phosphate gel step[7,13,23] was modified slightly by using in the first adsorption step 5.5 ml of gel per 1000 mg protein; in the second adsorption step 1.2 times the amount of gel used in the first adsorption step; and in the third adsorption step 0.4 times. The gel pellets were eluted in two steps with solutions containing 0.02 M potassium phosphate, 0.02 M NaCl and 0.02 M glycine, and 0.15 M potassium phosphate, 0.1 M NaCl and 0.1 M glycine (pH 7.8). The eluates were precipitated with solid $(NH_4)_2SO_4$ to 60% saturation. The DEAE-cellulose chromatography was carried out as described previously[23], except that a 5 cm × 35 cm column was used, and all buffer volumes and fraction volumes were increased correspondingly.

The DEAE-cellulose enzyme pool was concentrated by ultrafiltration to a final volume of 3–4 ml, and was then applied to a 2.5 cm × 90 cm column of Bio-Gel A-1.5 m, which was equilibrated and eluted with 0.2 M NaCl, 0.2 M glycine and 0.01 M Tris–HCl buffer (pH 7.8). The column was eluted with a flow rate of 20 ml/h, and 3-ml fractions were collected. The peak enzyme activity was eluted in Fractions 81 and 82. Fractions 70–84 (see Fig. 1) were pooled and used to constitute the purified enzyme preparation.

Enzymic reaction and assay procedures

The enzymic reaction under standard conditions was carried out in a final volume of 4 ml containing 10–1000 μg/ml enzyme protein (depending on the purity of the enzyme preparation), 125 μg/ml $(Pro–Gly–Pro)_n$, 0.08 mM $FeSO_4$, 0.5 mM α-ketoglutarate, 2 mM ascorbic acid, 0.2 mg/ml catalase (ref. 9), 0.1 mM dithiothreitol, 2 mg/ml bovine serum albumin (ref. 26), and 0.05 M Tris–HCl buffer adjusted to pH 7.8 at 25°. The samples were incubated at 37° for 1 h as described earlier[9], and, after hydrolysis, the amount of hydroxyproline synthesized was assayed[27]. In experiments in which the hydroxylation of [^{14}C]lysine was studied, the enzymic reaction was carried out as described above, with the exception that $(Pro–Gly–Pro)_n$ was replaced with 300 000 disint./min per 4 ml of [^{14}C]lysine-labelled protocollagen (see *Materials*). The [^{14}C]lysine and hydroxy[^{14}C]lysine contents were assayed by hydrolysing the samples and then chromatographing the samples on the short column of a Beckman Model 120 C amino acid analyser[16]. 0.5-min fractions (about 0.5 ml) were collected and assayed in a liquid-scintillation counter[28]. The protein content of the enzyme preparations was measured by peptide absorbance at 225 mμ, using serum albumin as a standard, and by ninhydrin assays of acid hydrolysates.

The sedimentation coefficient of the enzyme preparation was measured using a Spinco Model E analytical ultracentrifuge equipped with a split-beam photoelectric scanner accessory, in conjunction with a monochromator and ultraviolet absorption optical system. Before the experiment, the enzyme preparation was dialysed against a solution containing 0.05 M NaCl, 0.1 M glycine, 0.1 mM dithiothreitol and 0.01 M Tris–HCl buffer (pH 7.8). The protein content of the enzyme preparation was 0.20 or 0.40 mg/ml, the rotor speed 42 040 rev./min, and the temperature 23°. The sedimentation coefficient was corrected to values in water at 20°.

Disc electrophoresis was carried out in a 6.5% polyacrylamide gel with Tris–glycine buffer[29]. The pH of the upper buffer was 9 and that of lower buffer 8.2. The protein was stained with amido black or coomassie blue. For densitometry, the gels

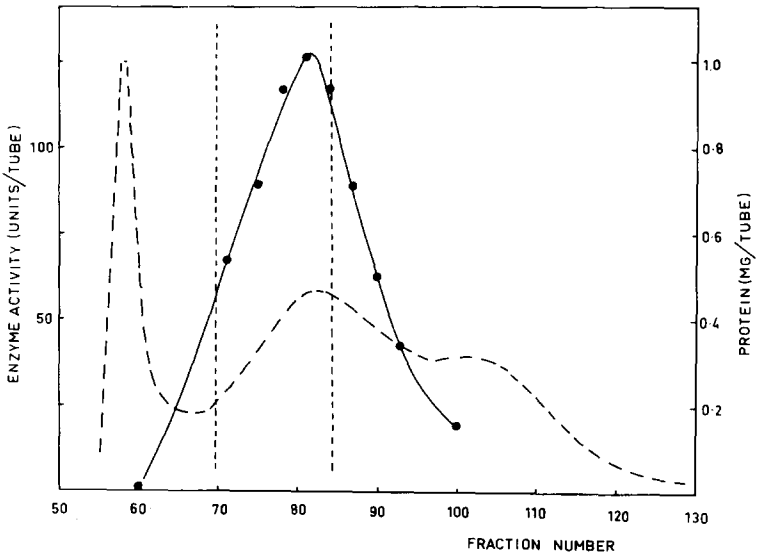


Fig. 1. Gel filtration of an enzyme preparation purified through the DEAE-cellulose chromatography step on a Bio-Gel A-1.5 m column. Solid line, enzymic activity (μg hydroxyproline synthesized under standard assay conditions per fraction); broken line, protein (mg/fraction). Fractions 70–84 were pooled as indicated by the vertical lines, and constituted the purified enzyme preparation.

were photographed, and the film was analysed with a Beckman Model RB Analytrol densitometer.

The amino acid analysis of the enzyme preparation was performed on a Beckman Model 120 C amino acid analyser. Before analysis the enzyme preparation was dialysed for three days against running tap water and then for four days against distilled water to remove the free glycine used in the buffers. The preparation was then lyophilized and hydrolysed with 1.0 ml of 6 M HCl for 22 h at 110° under a vacuum in sealed tubes.

RESULTS

Isolation of the purified enzyme

The purification procedure consisted of fractionation with $(NH_4)_2SO_4$, calcium phosphate gel fractionation (which included fractionating adsorption to the gel, fractionating elution of the gel pellets and $(NH_4)_2SO_4$ precipitation), chromatography on DEAE-cellulose, and gel filtration on an 8% agarose gel (Table I). In the Bio-Gel A-1.5 m gel-filtration step, the specific activity of the enzyme agreed within 10% in 15 fractions (Fig. 1, Fractions 70–84). This enzyme preparation synthesized about 300 μg hydroxyproline per mg protein per h in standard incubation conditions with 125 μg/ml of the polytripeptide substrate (Table I); and about 600 μg hydroxyproline per mg protein per h with a saturating concentration of the polytripeptide substrate. Three other similarly purified enzyme preparations synthesized about 200, 250, and 350 μg hydroxyproline per mg protein per h in standard incubation con-

TABLE I

PURIFICATION OF PROTOCOLLAGEN PROLINE HYDROXYLASE

The starting material was 15 000 × *g* supernatant of the homogenate of 1400 g of 13-day-old chick embryos. 1 unit of enzymic activity is defined as the amount of enzyme required to synthesize 1 μg hydroxyproline in 1 h with 125 μg/ml of $(Pro–Gly–Pro)_n$ as substrate.

Enzyme fraction	*Total protein (mg)*	*Total units of enzyme*	*Specific activity (units/mg)*
15 000 × *g* supernatant	84 700	—	(0.03–0.1)*
$(NH_4)_2SO_4$	23 700	23 700	1.0
Calcium phosphate gel	910	13 600	15
DEAE-cellulose	46	4 200	91
Biol-Gel A-1.5 m	8	2 300	290

* According to PROCKOP *et al.*[8].

ditions. One of these preparations was subjected to a second agarose gel filtration, but the specific activity only increased by about 10%.

Because the 15 000 × *g* supernatant of the embryo homogenates contains inhibitory substances[9], the exact degree of purification is difficult to express. However, the purification increased the specific activity of the enzyme several thousand times compared with the values estimated in the starting material, and about 300 times compared with the values in the first $(NH_4)_2SO_4$ fraction (Table I). These purified enzyme preparations retained over 80% of their activity after storage without thawing for 1 month at −20°.

Purity of the enzyme and preliminary estimation of the molecular weight

Disc electrophoresis of the purified enzyme preparation showed only two bands: one major band and one minor band to the anode (Fig. 2). When the fractions which eluted from the Bio-Gel A-1.5 m column behind the enzyme peak were analysed by disc electrophoresis, the minor band was much stronger than that in Fig. 2, and the major band was weaker. Two other enzyme preparations synthesizing 250 or 350 μg hydroxyproline per mg protein per h contained a similar major band and two minor bands on the cathodal side of the major band, whereas the minor band seen in Fig. 2 was absent.

Attempts to identify the enzyme activity from unstained gel were made by slicing the gels, and using homogenized gel slices as sources of the enzyme. [^{14}C]-Proline-labelled protocollagen, which is a more sensitive substrate for the enzyme than $(Pro–Gly–Pro)_n$ (ref. 9), was used as substrate in the incubation, and the amount of hydroxy[^{14}C]proline formed per 50 000 disint./min [^{14}C]proline-labelled protocollagen was assayed[30]. In one experiment no enzyme activity was found in any of the slices, whereas in two other experiments significant enzyme activities were recovered in the slices corresponding to the major band (Band I, Fig. 3), and no enzyme activity in other slices. These findings indicate that the major band represents the enzyme and the minor bands contaminants. Densitometry of Fig. 2 indicated that only about 5% of the total protein in the enzyme preparation is a contaminant, provided that the two proteins stain in a similar way with amido black.

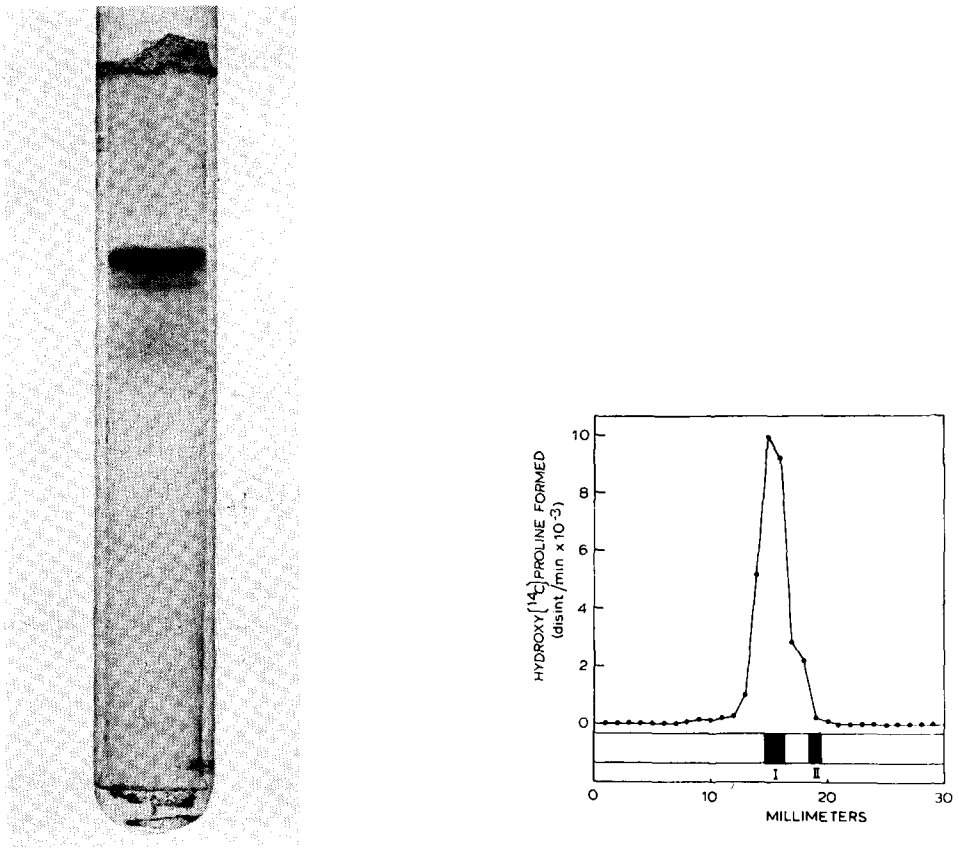
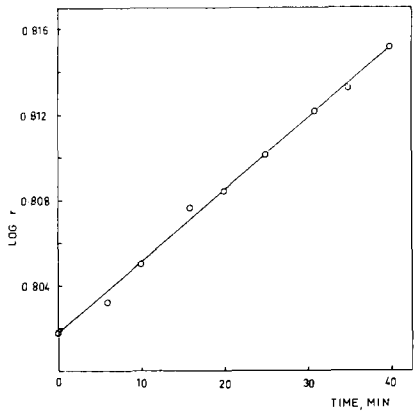


Fig. 2. Disc electrophoresis of the purified enzyme preparation synthesizing 290 μg hydroxyproline per mg protein per h in standard incubation conditions. The gel was stained with amido black. Anode at the bottom.

Fig. 3. Location of protocollagen proline hydroxylase activity in disc gels. 30 μg of purified enzyme were used for disc electrophoresis. The enzyme activity was assayed as described in the text. The activities are expressed as disint./min hydroxy[^{14}C]proline synthesized per 1 mm gel slice. The stained control gel, corrected for swelling during staining, is shown schematically under the graph.

No additional bands were seen when the gels were stained with coomassie blue, and the relative amounts of the two proteins seemed to be similar to those in Fig. 2.

The sedimentation of two enzyme preparations has been studied in the analytical ultracentrifuge. The enzyme preparation synthesizing 290 μg hydroxyproline per mg protein per h (Table I) gave only one component when analyzed at 280 mμ with the photoelectric scanning accessory. The plot of the sedimentation boundary was linear with time (Fig. 4), and the sedimentation coefficient was 6.7 S. The enzyme preparation synthesizing 250 μg hydroxyproline per mg protein per h gave a similar major component, and one additional minor component which sedimented more rapidly. The sedimentation coefficient suggests a molecular weight of about 150 000, if the enzyme is a fairly symmetrical globular protein with a normal amount



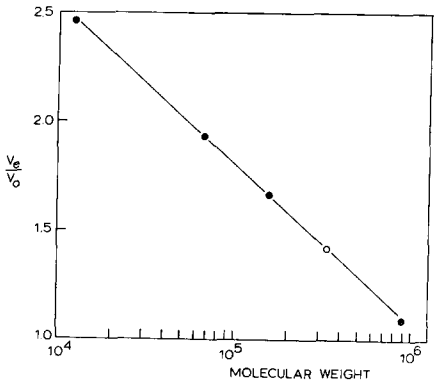


Fig. 4. Determination of the sedimentation coefficient of the purified enzyme preparation.

Fig. 5. Estimation of the molecular weight of the purified enzyme preparation by gel filtration on a Bio-Gel A-1.5 m column. Solid symbols: cytochrome *c*, bovine serum albumin, caeruloplasmin, and IgM globulin. Open symbol: the enzyme.

of hydration. On the other hand, the elution pattern of the enzyme in gel filtration suggests a molecular weight of about 350 000 (Fig. 5).

TABLE II

AMINO ACID COMPOSITION OF THE PURIFIED PROTOCOLLAGEN PROLINE HYDROXYLASE PREPARATION

The values, given with the accuracy of five residues, are means of determinations on three separate samples of the most highly purified enzyme preparation. Each sample, containing 0.2 mg protein, was dialysed, hydrolysed and analysed separately. The values for most amino acids agreed within ± 10% or ± five residues, but the value for serine in one analysis was 141, and in two other analysis about 100. The highest value was assumed to be due to a contaminant, and only the two latter values were used in the calculation. The values have not been corrected for losses during 22-h acid hydrolysis, and the values for tryptophan and cystine have not been determined.

Amino acid	*Residues per 1000 residues*
Hyp	0
Asp	95
Thr	50
Ser	100
Glu	180
Pro	35
Gly	100
Ala	75
Val	50
Met	15
Ile	35
Leu	65
Tyr	20
Phe	30
Lys	85
His	25
Arg	35

Amino acid composition of the purified enzyme preparation

Amino acid analysis of the purified protocollagen proline hydroxylase preparation was done to find out whether any unusual features would appear. Because the enzyme was not completely pure, the results are given only to the nearest five residues (Table II). The analysis suggests that the enzyme contains relatively large amounts of aspartic acid, glutamic acid, serine, glycine and lysine.

Effect of thiol reagents and diisopropylfluorophosphate on the activity of the purified enzyme

RHOADS *et al.*[26] reported a stimulation, by bovine serum albumin and dithiothreitol, of about 15-fold of the activity of partially purified protocollagen proline hydroxylase preparations from the skin of newborn rats. We confirmed these findings with our purified enzyme preparation, although the degree of stimulation was only about 1.5–3-fold (not shown). Because dithiothreitol was found to increase the activity of the purified protocollagen proline hydroxylase preparations, the effect of sulphydryl reagents on the activity of the enzyme was studied in an incubation system not containing dithiothreitol or bovine serum albumin (Table III). The enzyme was found to be effectively inhibited by *p*-mercuribenzoate; a 0.002-mM concentration gave 36% inhibition. After pre-incubation with 0.2 mM *p*-mercuribenzoate, most of the inhibition could still be reversed by dithiothreitol. Inhibition of the enzyme was also observed with *N*-ethylmaleimide, but about 200-fold concen-

TABLE III

EFFECT OF *p*-MERCURIBENZOATE, *N*-ETHYLMALEIMIDE AND DIISOPROPYLFLUOROPHOSPHATE ON PURIFIED PROTOCOLLAGEN PROLINE HYDROXYLASE

Purified protocollagen proline hydroxylase, 50 μg (Expts. I and III) or 60 μg (Expt. II), was preincubated for 10 min (Expts. I and II) or 20 min (Expt. III) at 37° in 2.0 ml of 0.05 M Tris–HCl buffer (pH 7.8) at 25°. *p*-Mercuribenzoate, *N*-ethylmaleimide, or diisopropylfluorophosphate were present during preincubation in the concentrations indicated. After preincubation, $(Pro–Gly–Pro)_n$ and the cofactors or cosubstrates were added, and incubation was continued in a final volume of 4.0 ml for 1 h under standard conditions, except that dithiothreitol and bovine serum albumin were not added. The stock solutions of *p*-mercuribenzoate, *N*-ethylmaleimide and diisopropylfluorophosphate were prepared immediately before use.

Expt. No.	*Addition*	*Hydroxyproline formed (μg)*	*Inhibition (%)*
I	None	3.9	0
	0.20 mM *N*-ethylmaleimide	4.1	0
	0.20 mM *p*-mercuribenzoate	0.8	80
	0.20 mM *p*-mercuribenzoate + 0.40 mM dithiothreitol*	3.0	23
II	None	5.2	0
	0.50 mM *N*-ethylmaleimide	3.5	33
	0.01 mM *p*-mercuribenzoate	2.2	58
III	None	4.2	0
	0.01 mM *p*-mercuribenzoate	1.0	76
	0.002 mM *p*-mercuribenzoate	2.7	36
	0.20 mM diisopropylfluorophosphate	4.2	0

* Dithiothreitol was added after the preincubation.

tration of the reagent was required for an inhibition comparable to that obtained with *p*-mercuribenzoate (Table III).

Because the enzyme preparation contained relatively large amounts of serine, the effect of diisopropylfluorophosphate was studied. No inhibition was observed with a concentration of 0.2 mM (Table III), indicating that the enzyme was not specifically inhibited by this compound.

Hydroxylation of lysine in protocollagen by the purified enzyme

To find out whether a single enzyme is responsible for the hydroxylation of both proline and lysine residues in protocollagen, a purified enzyme preparation was tested for its ability to hydroxylate [^{14}C]lysine in protocollagen. The results indicated that although the partially purified enzyme preparations did hydroxylate lysine, the purified enzyme preparation did not synthesize significant amounts of hydroxy[^{14}C]-lysine from [^{14}C]lysine (Table IV). Another purified preparation of protocollagen proline hydroxylase was likewise found not to hydroxylate lysine residues in protocollagen.

TABLE IV

HYDROXYLATION OF [^{14}C]LYSINE IN PROTOCOLLAGEN WITH DIFFERENT PREPARATIONS OF PROTOCOLLAGEN PROLINE HYDROXYLASE

[^{14}C]Lysine-labelled protocollagen, 300 000 disint./min, was incubated with different enzyme preparations under standard conditions, except that $(Pro–Gly–Pro)_n$ was not added. 1 unit of enzymic activity is defined as the amount of enzyme required to synthesize 1 μg hydroxyproline in 1 h with 125 μg/ml of $(Pro–Gly–Pro)_n$ as substrate.

Enzyme preparation	*Specific activity of enzyme for $(Pro–Gly–Pro)_n$ (units/mg)*	*Units of enzyme added*	*Hydroxy[^{14}C]lysine formed (disint./min)**
Enzyme omitted	—	0	0
$(NH_4)_2SO_4$	1.0	30	9120
Calcium phosphate gel	15	30	9410
DEAE-cellulose	75	60	7950
Bio-Gel A-1.5 m	250	60	220

* Values indicate disint./min hydroxy[^{14}C]lysine synthesized with 300 000 disint./min [^{14}C]lysine-labelled protocollagen as substrate.

DISCUSSION

With the purification procedure described in the present study, relatively stable preparations of highly purified protocollagen proline hydroxylase were obtained. The final enzyme preparation was homogeneous in the analytical ultracentrifuge, and showed only one minor contaminant in disc electrophoresis. Characterization of the protein by amino acid analysis indicated that the enzyme contained large amounts of acidic amino acids, and the acidic character of the protein was borne out by the relatively strong binding to DEAE-cellulose. Preliminary estimation of the molecular weight suggested a value of 150 000–350 000, indicating that the enzyme is a relatively large protein, probably consisting of subunits. The discrepancy

observed between the sedimentation coefficient and the molecular weight estimated by gel filtration may indicate that the enzyme is an asymmetric molecule. However, more exact physicochemical data on the pure enzyme are needed to determine the shape and size of the molecule. Another possibility is that the enzyme can exist either as monomers or dimers depending on the conditions.

Exact comparison of the specific activity of the purified enzyme with the values obtained by previous procedures is difficult, because the assay conditions were not identical. RHOADS AND UDENFRIEND[20] reported that their enzyme preparation, purified from the skin of newborn rats, synthesized 200–250 mμmoles (about 30 μg) of hydroxyproline per mg protein per h with a saturating concentration of (Pro–Gly–Pro)$_n$ of average molecular weight 4100. Recalculations from the data by KIVIRIKKO AND PROCKOP[16] suggest that their best enzyme preparations, purified from chick embryo extracts, would have synthesized about 100 μg hydroxyproline per mg protein per h under the present assay conditions with a saturating concentration of the polytripeptide substrate. Both these values are considerably lower than the 600 μg of hydroxyproline per mg protein per h obtained with our purified enzyme.

Calculation of the catalytic constant for the enzyme with (Pro–Gly–Pro)$_n$ as substrate gives a value of about 10–30 moles hydroxyproline formed per mole enzyme per min, depending on the molecular weight of the enzyme used in the calculations. This value is very low compared with those reported for most other enzymes. However, it should be noted that the polytripeptide (Pro–Gly–Pro)$_n$ is a less appropriate substrate for the enzyme than protocollagen (see refs. 9 and 14). The K_m value for the polytripeptides used in the present study is about 100 μg/ml (ref. 9), whereas that for protocollagen is about 1 μg/ml (ref. 16). The catalytic constant may therefore be higher when determined with the natural substrate.

The present data demonstrate effective inhibition of the purified enzyme by thiol reagents, and the reversibility of this inhibition by dithiothreitol. These results suggest that free sulphydryl groups are required for the activity of the enzyme. Thus dithiothreitol, which stimulates the enzyme[26], probably acts by generating these groups*. The experiment with diisopropylfluorophosphate suggests that even though the enzyme seems to contain relatively large amounts of serine, the hydroxyl groups of serine are possibly not required for the enzymic activity.

The experiments on the hydroxylation of [^{14}C]lysine-labelled protocollagen clearly demonstrate that the most highly purified protocollagen proline hydroxylase preparations did not hydroxylate lysine residues in protocollagen. Recent studies with other types of experiments have likewise suggested that proline and lysine are not hydroxylated at the same enzymic site, and probably not by the same enzyme[32]. It thus seems that two separate enzymes, protocollagen proline hydroxylase and protocollagen lysine hydroxylase, which require the same cofactors or cosubstrates, are involved in the hydroxylation of proline and lysine in protocollagen.

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* After the submission of our manuscript, POPENOE *et al.*[31] reported data on the effect of sulphydryl reagents on the activity of protocollagen proline hydroxylase, and their results support these conclusions.

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