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Partial Purification and Characterization of Chick-Embryo Prolyl 3-Hydroxylase

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Prolyl 3-hydroxylase was purified up to about 5000-fold from an $(NH_4)_2SO_4$ fraction of chick-embryo extract by a procedure consisting of affinity chromatography on denatured collagen linked to agarose, elution with ethylene glycol and gel filtration. The molecular weight of the purified enzyme is about 160000 by gel filtration The enzyme is probably a glycoprotein, since (*a*) its activity is inhibited by concanavalin A, and (*b*) the enzyme is bound to columns of this lectin coupled to agarose and can be eluted with a buffer containing methyl α-D-mannoside. The K_m values for Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate in the prolyl 3-hydroxylase reaction were found to be very similar to those previously reported for these co-substrates in the prolyl 4-hydroxylase and lysyl hydroxylase reactions.

Prolyl 3-hydroxylase catalyses the synthesis of 3-hydroxyproline in collagen by the hydroxylation of prolyl residues in peptide linkages, probably in the sequences -Gly-Pro-4Hyp-Gly- (Risteli *et al.*, 1977; Tryggvason *et al.*, 1977). The reaction requires Fe^{2+} ions, molecular oxygen, 2-oxoglutarate and ascorbate, and is further affected by the chain length and conformation of the polypeptide substrate (Risteli *et al.*, 1977, 1978*a*). Prolyl 3-hydroxylase and prolyl 4-hydroxylase activities have been demonstrated to be attributable to separate enzyme proteins (Tryggvason *et al.*, 1977), but no attempts to purify the 3-hydroxylase have yet been reported. Only a few studies are available on changes in prolyl 3-hydroxylase activity in vertebrate tissues (Risteli *et al.*, 1978*b*; Tryggvason *et al.*, 1978, 1979).

Prolyl 3-hydroxylase was purified here up to about 5000-fold from an $(NH_4)_2SO_4$ fraction of chick-embryo extract by an affinity-chromatography procedure, and the enzyme protein and the reaction with the enzyme were then partially characterized.

Experimental

Materials

Fertilized eggs of white Leghorn chickens were purchased from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland), and incubated at 37°C in a moist atmosphere for 14 days. Collagen, soluble in 0.075M-sodium citrate buffer, pH3.7, was prepared from rat skin (Gallop & Seifter, 1963) and coupled to agarose as previously described (L. Risteli *et al.*, 1976) by using a CNBr activation technique (Cuatrecasas & Anfinsen, 1971). Sepharose 4B and concanavalin A–Sepharose 4B (concanavalin A–agarose) were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden) and Bio-Gel A-1.5m (200-400 mesh) was from Bio-Rad Laboratories (Richmond, CA, U.S.A.). [2,3-^{3}H]Proline (specific radioactivity 35 Ci/mmol) was from New England Nuclear Corp. (Boston, MA, U.S.A.).

Purification of prolyl 3-hydroxylase

All procedures were carried out at 0–4°C

Preparation of initial extract. A total of 400 14-day chick embryos were homogenized twice for 30s with a 1-min interval in a Waring blender at full speed in batches of about 30 embryos in a solution containing 0.2M-NaCl, 0.1M-glycine, 0.1% (w/v) Triton X-100, 1mM-β-mercaptoethanol and 50mM-Tris/HCl buffer adjusted to pH7.5 at 4°C (1ml of solution/g of embryos). The homogenate was left with occasional stirring for about 1h and was then centrifuged at 15000*g* for 30min.

$(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was slowly stirred into the supernatant fraction to a final concentration of 55% saturation. The pellet obtained by centrifugation at 15000*g* for 20min was dissolved in a solution containing 0.2M-NaCl, 0.1M-glycine, 1mM-β-mercaptoethanol and 50mM-Tris/HCl buffer, adjusted to pH7.5 at 4°C (termed here enzyme buffer). The preparation was dialysed for 4h against 20 litres of this solution and for 12h against another 20 litres, and then centrifuged at 15000*g* for 20min to remove a small amount of insoluble material. The preparation was stored in 100–500ml batches at −20°C.

Collagen–agarose chromatography. A portion of the $(NH_4)_2SO_4$ fraction was thawed and centrifuged at 15000*g* for 20min to remove insoluble material. The supernatant, at a protein concentration of about 20mg/ml, was passed through an affinity column

with a bed volume of either 6ml (1cm×8cm; for up to 150ml of the sample) or 40ml (2cm×13cm; for up to 1000ml) at a flow rate of about 1 column volume/h. The column was washed with the enzyme buffer until the A_{225} of the eluate was less than 0.05. The enzyme was then eluted with the buffer containing 50% (v/v) ethylene glycol. The fractions were assayed for protein and prolyl 3-hydroxylase activity, and those with the highest specific activities were pooled. This pool was diluted with 4vol. of the enzyme buffer and concentrated in an Amicon ultrafiltration cell with a PM-30 membrane to a volume of about 2ml.

Gel filtration. The concentrated sample was applied to a Bio-Gel A-1.5m column (1.5cm×86cm) equilibrated with the enzyme buffer. The column was eluted with the same solution, and 3.5ml fractions were collected.

Chromatography on a concanavalin A–agarose column

A sample of the $(NH_4)_2SO_4$ fraction was thawed and centrifuged at 15000*g* for 20min at 4°C to remove insoluble material. A portion of the supernatant (50–100ml; protein concn. about 15mg/ml) was applied at a flow rate of 5–10ml/h to a 10ml column of concanavalin A-agarose equilibrated with the enzyme buffer. The column was washed until the A_{225} of the eluate was less than 0.05. The column was eluted with the enzyme buffer containing 1M-1-*O*-methyl α-D-mannoside, and 3.5ml fractions were collected.

Assays

Prolyl 3-hydroxylase activity was assayed by using a method based on the release of ^3H_2O during 3-hydroxylation of a [2,3-^{3}H]proline-labelled biologically prepared polypeptide substrate in which all prolyl residues recognized by prolyl 4-hydroxylase had been converted into 4-hydroxyprolyl residues (Risteli *et al.*, 1978*a*). In all experiments with crude enzyme preparations and in those in which the degree of purification of the enzyme was to be determined, the incubation with the enzyme was carried out at 20°C for 30min (Risteli *et al.*, 1977, 1978*a*). In experiments on the properties of the purified enzyme the incubations were carried out at 30°C for 30min.

The protein content of the enzyme preparations was measured by peptide absorbance at 225nm by using bovine serum albumin as a standard, which gave an absorbance coefficient of $A_{225}^{1mg/ml}=7.40$ with a 1cm light-path.

Results

Partial purification of the enzyme

The α chains of type-I collagen contain a number of -Gly-Pro-4Hyp-Gly- sequences (Fietzek & Kühn, 1976), the probable substrate sequences for prolyl 3-hydroxylase (Risteli *et al.*, 1977; Tryggvason *et al.*, 1977). Attempts were therefore made to purify the enzyme by using an affinity column in which denatured citrate-soluble rat skin collagen was linked

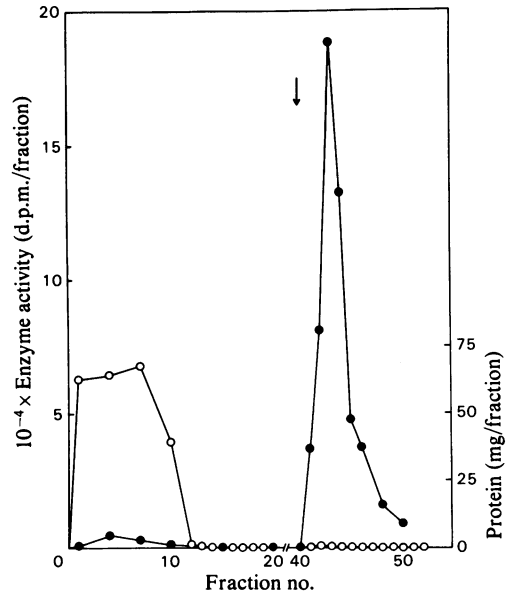

Fig. 1. *Chromatography of chick-embryo prolyl 3-hydroxylase on a collagen–agarose column*

A 60ml portion (21mg of protein/ml) of chick-embryo enzyme precipitated by $(NH_4)_2SO_4$ was passed through a 6ml collagen–agarose column in the enzyme buffer as described in the Experimental section. The column was then washed with the enzyme buffer and eluted with the same buffer containing 50% ethylene glycol (arrow). The fractions were of 10ml until tube 39, and 2.2ml thereafter. ●, Enzyme activity; ○, protein.

Table 1. *Partial purification of prolyl 3-hydroxylase from a chick-embryo $(NH_4)_2SO_4$ preparation*

One unit of enzyme activity is defined as the amount of enzyme present in 1mg of $(NH_4)_2SO_4$-precipitated chick-embryo extract.

Enzyme fraction	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg)
$(NH_4)_2SO_4$, 0–55% satn.	18400	18400	100	1
Collagen–agarose	6.58	8000	43	1216
Gel filtration	1.02	4360	24	4270*

* Fraction with the highest specific activity contained 5240 units/mg.

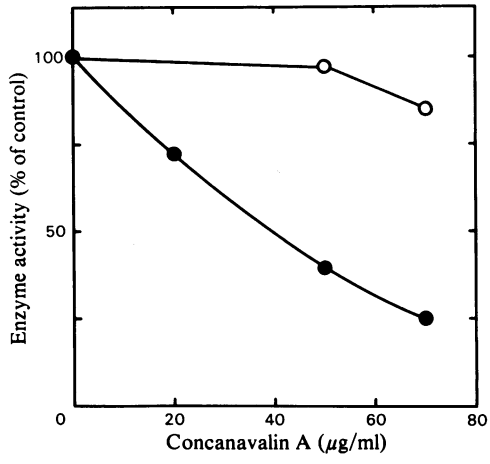

Fig. 2. *Inhibition of prolyl 3-hydroxylase activity by concanavalin A*

The reaction with an enzyme purified about 4000-fold was carried out as described in the Experimental section with various concentrations of concanavalin A in the absence (●) and presence (○) of 40mM-methyl α-D-mannoside. The enzyme activity is expressed as a percentage of that found in the absence of concanavalin A.

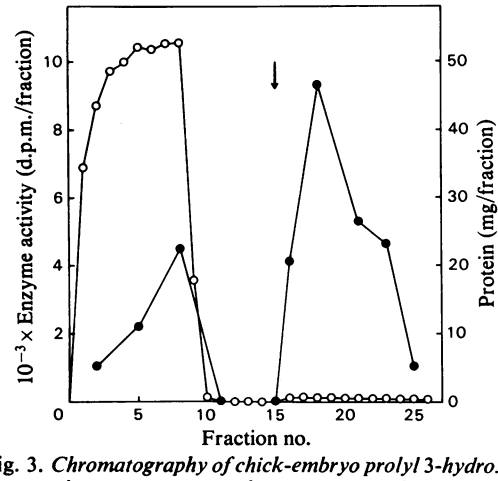

Fig. 3. *Chromatography of chick-embryo prolyl 3-hydroxylase on a concanavalin A–agarose column*

Chick-embryo enzyme precipitated by $(NH_4)_2SO_4$ (65ml; 14.5mg/ml; specific activity 370d.p.m./mg) was passed through a 10ml column and then washed with the enzyme buffer as described in the Experimental section. The enzyme was eluted with the enzyme buffer containing 1.0M-methyl α-D-mannoside (arrow). ●, Enzyme activity; ○, protein.

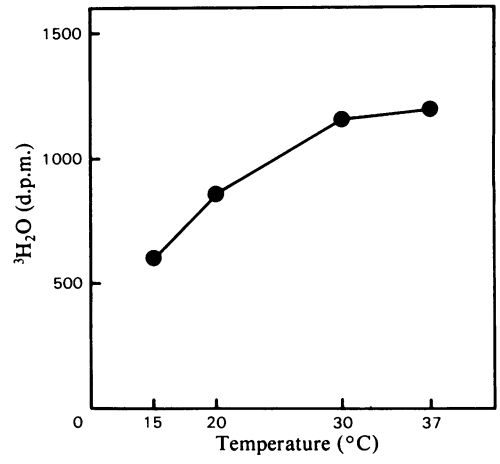

Fig. 4. *Effect of temperature on the formation of ^3H_2O in the prolyl 3-hydroxylase reaction*

A portion (1000000d.p.m.) of [2,3-^{3}H]proline-labelled fully 4-hydroxylated chick-embryo tendon protocollagen was used as a substrate. An enzyme purified about 4000-fold was incubated with the substrate for 30min at different temperatures as described in the Experimental section. Results are expressed as d.p.m. of ^3H_2O formed.

to agarose. The enzyme became efficiently bound to such columns (Fig. 1), and could be eluted with the enzyme buffer containing 50% ethylene glycol (Anttinen *et al.*, 1977). These findings made it possible to develop a simple purification procedure for prolyl 3-hydroxylase, which consisted of affinity chromatography on collagen–agarose and gel filtration (Table 1). The enzyme pool obtained was about 4200-fold purified over the original $(NH_4)_2SO_4$-fractionated enzyme, and the highest specific activity observed in a single fraction from the gel-filtration column corresponded to a purification of about 5200-fold. The enzyme was still not pure, however, as six bands were seen when the preparation was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Weber & Osborn, 1969) after reduction with β-mercaptoethanol (results not shown).

Properties of the enzyme protein

The molecular weight of the purified enzyme was about 160000 when determined by gel filtration in a Bio-Gel A-1.5m column calibrated with Blue Dextran, thyroglobulin (mol.wt. 660000), alcohol dehydrogenase (mol.wt. 150000), bovine serum albumin (mol.wt. 68000) and cytochrome *c* (mol.wt. 11700). Its activity was inhibited by concanavalin A, and this inhibition was reversed in the presence of methyl α-D-mannoside (Fig. 2). This suggested the existence of carbohydrate units on the enzyme molecule. Further support for this was obtained

Table 2. *K_m values for the co-substrates of the prolyl 3-hydroxylase reaction*

The K_m values were determined as described in the legend to Fig. 5. Previously reported values for prolyl 4-hydroxylase with protocollagen (Kivirikko & Prockop, 1967) and a synthetic substrate of the structure (Pro-Pro-Gly)$_5$ or (Pro-Pro-Gly)$_{10}$ (Myllylä *et al.*, 1977*b*) are shown for comparison. Abbreviation: n.d., not determined.

Co-substrate	3-Hydroxylase Biological substrate (μM)	4-Hydroxylase Biological substrate (Kivirikko & Prockop, 1967) (μM)	4-Hydroxylase Synthetic substrate (Myllylä *et al.*, 1977*b*) (μM)
Fe^{2+}	2	2	4
2-Oxoglutarate	3	5	22
O_2	30	n.d.	40
Ascorbate	120	100	300

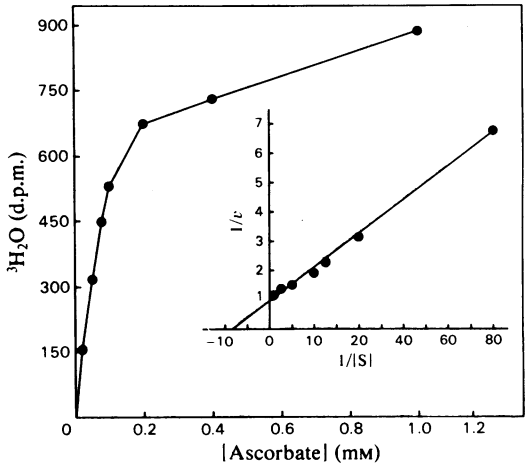

Fig. 5. *Effect of ascorbate concentration on the rate of the prolyl 3-hydroxylase reaction*

The reaction was carried out at 30°C for 30min as described in the Experimental section and the radioactivity of the ^3H_2O formed was determined. The K_m was 120μM as calculated from the regression line ($r = 0.94$). In the double-reciprocal plot (inset) the values shown on the abscissa are reciprocal values for the ascorbate concentration ([S], mM) and those shown on the ordinate are reciprocal values of $10^{-3}\times$ the velocity [v, radioactivity of the product formed (d.p.m.) in 30min].

when crude $(NH_4)_2SO_4$-precipitated enzyme was chromatographed on concanavalin A-agarose (Fig. 3). About 90% of the enzyme activity became bound to the column, the specific activity in fractions 2, 5 and 8 being about 6, 11 and 23% of that of the $(NH_4)_2SO_4$-precipitated enzyme. The relatively high activity in fraction 8 was probably due to exceeding the column capacity. It was also possible to elute about 20–30% of the original activity with the enzyme buffer containing 1.0M-methyl α-D-mannoside (Fig. 3).

Properties of the enzyme reaction

Although the highest rate of 3-hydroxyproline formation with crude prolyl 3-hydroxylase from rat kidney cortex was previously found to occur at 20°C (Risteli *et al.*, 1977), the purified chick-embryo enzyme studied here gave higher reaction rates at 30°C and 37°C (Fig. 4). To avoid over-estimation of the enzyme purification, all experiments in which the degree of purification was studied were carried out at 20°C.

The K_m values for Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate have not been studied previously. These values were now determined for the purified enzyme (Table 2), as shown for ascorbate in Fig. 5. The values were very similar to those determined for prolyl 4-hydroxylase with a biologically prepared protocollagen substrate (Table 2).

Discussion

It is only relatively recently that prolyl 3-hydroxylase and 4-hydroxylase have been shown to be separate enzymes (Risteli *et al.*, 1977; Tryggvason *et al.*, 1977). The formation of 3-hydroxyproline, 4-hydroxyproline and hydroxylysine in the biosynthesis of collagen thus involves three separate enzymes. Prolyl 4-hydroxylase has been isolated as a homogeneous protein from three sources (Berg & Prockop, 1973; Tuderman *et al.*, 1975; Kuutti *et al.*, 1975; J. Risteli *et al.*, 1976; Chen-Kiang *et al.*, 1977), and lysyl hydroxylase has been purified several thousandfold from chick-embryo extract (Ryhänen, 1976; Turpeenniemi *et al.*, 1977), whereas no previous attempts to purify prolyl 3-hydroxylase have been reported.

The highest known activity of prolyl 3-hydroxylase is found in the kidney cortex of young rats, with quite

high activity also occurring in several tissues from chick embryos (Tryggvason *et al.*, 1979). As it is easier to obtain large quantities of chick embryos than kidney cortexes of newborn rats, a homogenate of whole chick embryos was chosen here as the starting material for enzyme purification. The present study indicates that prolyl 3-hydroxylase can be efficiently purified by using an affinity column in which denatured citrate-soluble rat skin collagen is linked to agarose, and by applying this information it was possible to purify the enzyme up to about 5000-fold by relatively simple steps. The enzyme was not pure, however, and final purification may be difficult, as this protein is probably present in tissue in very small quantities.

The molecular weight of the purified chick embryo enzyme was about 160000 by gel filtration, a value which is similar to that previously found for a crude enzyme from rat kidney cortex (Tryggvason *et al.*, 1977). This hydroxylase thus has a somewhat lower molecular weight than prolyl 4-hydroxylase, the value for which is about 240000 by sedimentation equilibrium (Pänkäläinen *et al.*, 1970; Berg & Prockop, 1973; Tuderman *et al.*, 1975), and lysyl hydroxylase, with a value of about 200000 by gel filtration (Turpeenniemi *et al.*, 1977).

Prolyl 3-hydroxylase appears to be a glycoprotein, as its activity is inhibited by concanavalin A and as the enzyme becomes bound to columns containing this lectin coupled to agarose and can be eluted with a buffer containing methyl α-D-mannoside. It has previously been reported that the four other intracellular enzymes of collagen biosynthesis, namely prolyl 4-hydroxylase (Guzman *et al.*, 1976), lysyl hydroxylase (Ryhänen, 1976; Turpeenniemi *et al.*, 1977), hydroxylysyl galactosyltransferase (Risteli, 1978) and galactosylhydroxylysyl glucosyltransferase (Anttinen *et al.*, 1977; Myllylä *et al.*, 1977*a*) are glycoproteins, and prolyl 3-hydroxylase appears to be similar in this respect.

The K_m values for the co-substrates of the prolyl 3-hydroxylase reaction were determined here for the first time. Only minor differences were found between these values and those previously reported for prolyl 4-hydroxylase (Kivirikko & Prockop, 1967; Myllylä *et al.*, 1977*b*) and lysyl hydroxylase (Kivirikko & Prockop, 1972), suggesting that the three enzymes are very similar in their catalytic properties.

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