# Isolation of lysyl hydroxylase, an enzyme of collagen synthesis, from chick embryos as a homogeneous protein

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Two procedures are reported for the purification of lysyl hydroxylase, both procedures involving $(NH_4)_2SO_4$ fractionation, affinity chromatography on concanavalin A–agarose and elution of the column with ethylene glycol. The additional steps in procedure A consist of gel filtration and chromatography on a hydroxyapatite column, and in procedure B of affinity chromatography on collagen linked to agarose and gel filtration. The best preparations obtained with either of the two procedures were pure when examined by sodium dodecyl sulphate/polyacrylamide-disc-gel or slab-gel electrophoresis, but about half of the preparations obtained by procedure A had minor contaminants. The specific activity of a typical preparation purified by procedure B was 13 400 times that of the 15 000 g supernatant of the chick-embryo homogenate, with a recovery of about 4%. The molecular weight of the pure enzyme was about 200 000 by gel filtration, and that of the enzyme subunit about 85 000 by sodium dodecyl sulphate/polyacrylamide-disc-gel or slab-gel electrophoresis. It is suggested that the active enzyme is a dimer consisting of only one type of monomer, and that a previously described enzyme form with an apparent molecular weight of about 550 000 is a polymeric form of this dimer. The catalytic-centre activity of the pure enzyme, as determined with a saturating concentration of a synthetic peptide substrate and under conditions specified, was about 3–4 mol/s per mol.

Hydroxylysine is found in vertebrate proteins only in collagens and a few other proteins with collagen-like amino acid sequences (for recent reviews, see Kivirikko & Risteli, 1976; Prockop *et al.*, 1979*a*,*b*; Kivirikko & Myllylä, 1980). The hydroxy groups of hydroxylysyl residues have two important functions: they serve as sites of attachment for carbohydrate units, either as the monosaccharide galactose or the disaccharide glucosylgalactose (see Kivirikko & Myllylä, 1979), and they are essential for the stability of the intermolecular collagen cross-links (see Bailey *et al.*, 1974).

The synthesis of hydroxylysine occurs as a post-translational modification by means of the hydroxylation of lysyl residues incorporated into peptide linkages. The reaction probably occurs within the cisternae of the rough endoplasmic reticulum, and is catalysed by lysyl hydroxylase (peptidyl-lysine, 2-oxoglutarate–oxygen 5-oxido-reductase, EC 1.14.11.4), an enzyme requiring $Fe^{2+}$, $O_2$, 2-oxoglutarate and ascorbate (see Kivirikko & Risteli, 1976; Prockop *et al.*, 1979*a*,*b*; Kivirikko & Myllylä, 1980). Kinetic analyses suggest that the enzyme operates through an ordered reaction mechanism (Puistola *et al.*, 1980*a*,*b*).

Lysyl hydroxylase was first purified several hundred- or thousand-fold from chick-embryo extract by procedures consisting of conventional protein-purification steps (Kivirikko & Prockop, 1972; Ryhänen, 1976). The enzyme was then found to have a very high affinity for columns of concanavalin A–agarose (Ryhänen, 1976), and could be eluted only with a combination of methyl $\alpha$-D-mannoside or methyl $\alpha$-D-glucoside and ethylene glycol (Turpeenniemi *et al.*, 1977). By applying this information, a procedure was developed for purifying the enzyme about 3000-fold from chick-embryo extract (Turpeenniemi *et al.*, 1977), but the enzyme has not previously been isolated as a homogeneous protein from any source.

In the work reported here, lysyl hydroxylase was isolated from chick embryos as a homogeneous protein by using two alternative procedures and the enzyme protein 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 humidified incubators. The [$^{14}$C]lysine-labelled protocollagen substrate was prepared in freshly isolated cells from the leg tendons of 17-day chick embryos (Risteli & Kivirikko, 1976), and the synthetic peptide substrate L-I (Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly) was purchased from the Protein Research Foundation (Minoh, Osaka, Japan). Citrate-soluble rat skin collagen was coupled to 4% agarose (Sepharose 4B; Pharmacia Fine Chemicals AB, Uppsala, Sweden) by the CNBr-activation technique, as described previously (Risteli et al., 1976), and the product was termed collagen–agarose. In most coupling experiments the collagen content was 1.5–4 mg/ml of packed gel, as measured by hydrolysing a sample of the gel and assaying the hydroxyproline on an amino acid analyser (see Risteli et al., 1976). Concanavalin A–agarose (concanavalin A–Sepharose 4B) was from Pharmacia, and hydroxyapatite (Bio-Gel HTP) and Bio-Gel A-1.5 m (200–400 mesh) were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). 1-O-Methyl α-D-glucoside was from Sigma (St. Louis, MO, U.S.A.) and ethylene glycol from Merck (Darmstadt, Germany). The sources of the radio-isotopes and other materials were as given previously (Puistola et al., 1980a,b).

## Purification of lysyl hydroxylase

All procedures were carried out at 0–4°C. The term 'enzyme buffer' is used for a solution containing 0.2 M-NaCl, 0.1 M-glycine, 10 μM-dithiothreitol, 1% glycerol and 20 mM-Tris/HCl buffer, adjusted to pH 7.5 at 4°C.

*Preparation of the $(NH_4)_2SO_4$-fractionated enzyme.* This enzyme from 14-day whole chick embryos was obtained as a fraction of the 15 000 g supernatant of the embryo homogenate precipitated with 17–55% $(NH_4)_2SO_4$ saturation (Ryhänen, 1976; Turpeenniemi et al., 1977). The final dialysis was against enzyme buffer supplemented with 3 mM-MnCl$_2$.

*Chromatography on a concanavalin A–agarose column.* The dialysed $(NH_4)_2SO_4$-fractionated enzyme was centrifuged at 15 000 g for 20 min, the supernatant was diluted to a protein concentration of about 20 mg/ml, and a sample of about 2000 ml was passed at a flow rate of 50–70 ml/h through a column of concanavalin A–agarose (3.8 cm × 4.5 cm; about 50 ml), equilibrated with enzyme buffer containing 3 mM-MnCl$_2$. The column was washed at the same flow rate with enzyme buffer containing 3 mM-MnCl$_2$ and 1 M-methyl α-D-glucoside until the $A_{225}$ of the eluate was about 0.1 (usually about 48 h), and the enzyme was then eluted with 1000 ml of enzyme buffer containing 0.3 M-methyl α-D-glucoside and

60% (v/v) ethylene glycol (Turpeenniemi et al., 1977).

*Further purification by procedure A.* The eluate from the previous step was concentrated to about 10 ml in an ultrafiltration cell (Amicon, Lexington, MA, U.S.A.) with a PM-30 membrane, and this sample was applied to a Bio-Gel A-1.5 m column of size 2.5 cm × 90 cm equilibrated and eluted with enzyme buffer. The fractions containing most of the enzyme activity were pooled and concentrated as described above to about 5 ml.

This pool was adjusted to 0.01 M with respect to potassium phosphate, pH 7.5, by adding an 0.1 M solution, and applied to a hydroxyapatite column (1.0 cm × 10 cm) equilibrated with enzyme buffer without Tris/HCl but containing 0.01 M-potassium phosphate, pH 7.5. The column was eluted in a stepwise manner with a solution containing 0.1 M-NaCl, 0.1 M-glycine, 10 μM-dithiothreitol, 1% glycerol and increasing concentrations of potassium phosphate, pH 7.5, as shown in Fig. 1. Most of the enzyme activity was eluted with a solution containing 0.18 M-potassium phosphate.

*Further purification of the concanavalin A–agarose enzyme pool by procedure B.* The eluate from the concanavalin A–agarose column was dialysed overnight against enzyme buffer and then applied at a flow rate of 20 ml/h to a collagen–agarose column (1.2 cm × 18 cm; about 20 ml), equilibrated with enzyme buffer. The column was washed at the same flow rate with 100 ml of enzyme buffer and then eluted with 160 ml of enzyme buffer containing 60% (v/v) ethylene glycol. The fractions were pooled as indicated in Fig. 2, and concentrated to about 2 ml in an Amicon ultrafiltration cell with a PM-30 membrane.

This pool was applied to a Bio-Gel A-1.5 m column (1.05 cm × 92 cm) equilibrated and eluted with enzyme buffer. The fractions containing most of the enzyme activity constituted the final enzyme pool.

## Assay of lysyl hydroxylase activity

In most experiments the enzyme activity was measured with [$^{14}$C]lysine-labelled protocollagen substrate, as described previously (Kivirikko & Prockop, 1972), except that the substrate was prepared in isolated chick-embryo tendon cells (see under 'Materials') and that the ascorbate concentration was 1 mM (Puistola et al., 1980a). In some experiments the activity was assayed with the synthetic peptide substrate L-I, this method being based on the measurement of the $^{14}CO_2$ formed during hydroxylation-coupled decarboxylation of 2-[1-$^{14}$C]oxoglutarate (Kivirikko et al., 1972). This assay was likewise carried out as described previously (Kivirikko et al., 1972), except that the

ascorbate concentration was 1 mM (Puistola et al., 1980a).

### Other assays

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

Disc electrophoresis of native enzyme was carried out with 7.5% polyacrylamide gels by using either a two-gel system or only one gel (see Tuderman et al., 1975). Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis (Weber & Osborn, 1969) or slab-gel electrophoresis (King & Laemmli, 1971) was carried out after denaturation and reduction of the polypeptide chains. The gels were stained with Coomassie Brilliant Blue.

## Results

### Purification of lysyl hydroxylase

Two different procedures for lysyl hydroxylase purification are reported here (Table 1), both involving affinity chromatography on concanavalin A–agarose and elution of the column with ethylene glycol, as reported previously (Turpeenniemi et al., 1977). The additional steps in procedure A (Table 1) consist of gel filtration and chromatography on a hydroxyapatite column, in which the enzyme activity is eluted as a single peak with 0.18 M-phosphate buffer (Fig. 1). In a typical case the procedure gave an increase in specific activity of about 8400-fold compared with the original chick-embryo extract, the recovery being only about 2% (Table 1). The highest specific activities seen with this pro-

cedure in any preparation were about 10 000 times those of the chick-embryo extract.

Procedure B (Table 1) involves a second affinity chromatography on collagen linked to agarose. It had previously been reported that crude chick-embryo lysyl hydroxylase does not become bound to collagen–agarose (Ryhänen, 1976). We now found, however, that an enzyme purified by affinity chromatography on concanavalin A–agarose becomes bound efficiently to columns of collagen–agarose
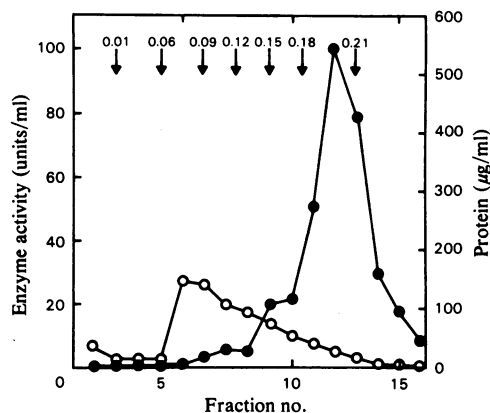


Fig. 1. Chromatography of lysyl hydroxylase on a hydroxyapatite column in purification procedure A
Conditions were as described in the Experimental section. Fractions (3.2 ml) nos. 12–17 were pooled and constituted the purified enzyme. The arrows indicate changes to buffers with the phosphate concentrations (M) indicated. ●, Enzyme activity (for units see Table 1); ○, protein.

Table 1. Purification of lysyl hydroxylase from a chick-embryo $(NH_4)_2SO_4$ fraction
One unit of enzyme activity is defined as the amount of enzyme present in 1 mg of the $(NH_4)_2SO_4$ fraction (17–55% saturation) obtained from the 15 000 g supernatant of chick-embryo homogenate (Kivirikko & Prockop, 1972; Ryhänen, 1976). This enzyme activity and those in the purified enzyme fractions were assayed with [$^{14}$C]lysine-labelled protocollagen substrate as described in the Experimental section. The purification was calculated on the basis of a specific activity of 0.5 unit/mg (Kivirikko & Prockop, 1972; Ryhänen, 1976; Turpeenniemi et al., 1977) for the original 15 000 g supernatant.

| Procedure and enzyme fraction | Total protein (mg) | Total activity (units) | Recovery (%) | Specific activity (units/mg) | Purification (-fold) |
|---|---|---|---|---|---|
| Procedure A | | | | | |
| Ammonium sulphate | 39 300 | 39 300 | 100 | 1.0 | 2 |
| Concanavalin A–agarose | 79 | 30 900 | 79 | 390 | 780 |
| Gel filtration | 4.0 | 6140 | 16 | 1540 | 3080 |
| Hydroxyapatite | 0.17 | 716 | 1.8 | 4210 | 8420 |
| Procedure B | | | | | |
| Ammonium sulphate | 43 800 | 43 800 | 100 | 1.0 | 2 |
| Concanavalin A–agarose | 91 | 34 200 | 78 | 380 | 760 |
| Collagen–agarose | 1.5 | 5010 | 11 | 3340 | 6680 |
| Gel filtration | 0.24 | 1610 | 3.7 | 6700 | 13 400 |

provided that the ethylene glycol has first been removed by dialysis. In typical experiments less than 15% of the enzyme applied to the column is found in the effluent (result not shown). The enzyme can be eluted with enzyme buffer containing 60% (v/v) ethylene glycol, most of the lysyl hydroxylase activity being separated from the main protein peak (Fig. 2). To obtain a high degree of purification, the fractions were pooled as indicated in Fig. 2, thus omitting in various experiments about one-quarter to one-third of the eluted enzyme. The enzyme activity was eluted in the final gel filtration as a single peak of constant specific activity (Fig. 3). The purification of a typical preparation was 13 400-fold, with a recovery of 4% (Table 1).

*Purity and properties of the enzyme*

The best enzyme preparations obtained with either of the two procedures gave only one band when studied by sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis in 10% polyacrylamide gels prepared with 0.5 times the normal amount of cross-linker (Weber & Osborn, 1979) (Fig. 4) or by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis with 8%-acrylamide gels (results not shown). Experiments carried out by overloading the gels demonstrated the absence of additional bands, and those carried out at very low protein concentrations showed that it was indeed a single band that was observed (results not shown).

About half of the preparations obtained by procedure A contained one or two additional bands representing 5–10% of the total protein, and some preparations obtained with procedure B had minor contaminants representing less than 5–10% of the total protein. It was not possible to study lysyl hydroxylase as a native protein by polyacrylamide-disc-gel electrophoresis, as most of the enzyme was precipitated at the top of the gel even when the experiments were carried out in the presence of 0.1% (w/v) Triton X-100.

The preparations obtained by procedure A lost about half of their activity in 1 day at +4°C or during one cycle of freezing and thawing. This rapid inactivation probably explains the lower specific activity of pure preparations obtained by procedure A than with procedure B. The enzyme activity in the ethylene glycol-containing fractions from the collagen–agarose column in procedure B was very stable, and the fractions, could be stored for several months at −20°C, a temperature at which they do not freeze, with little, if any, inactivation. There were usually large losses of activity (up to half), however, during concentration of the enzyme for the last gel filtration. The final purified enzyme lost about half of its activity in a few days at +4°C or during one cycle of freezing and thawing, whereas this loss did not differ greatly when the enzyme was stored frozen for a few days or months.

The molecular weight of the native enzyme was about 200 000 by gel filtration on a Bio-Gel A-1.5 m
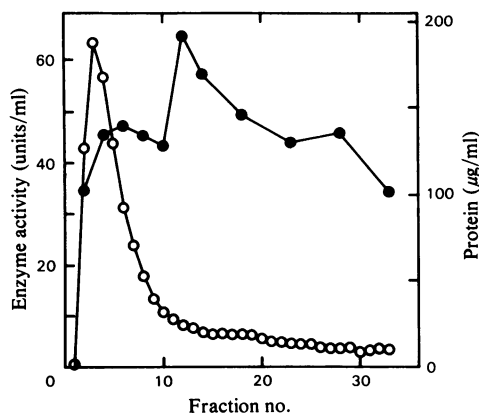


Fig. 2. *Elution of lysyl hydroxylase from the collagen–agarose column in purification procedure B*
The enzyme was applied to the column and the column washed as described in the Experimental section (results not shown). The enzyme was then eluted with 160 ml of enzyme buffer containing 60% ethylene glycol. Fractions (4.8 ml) nos. 12–33 were pooled and purified further. ●, Enzyme activity (for units see Table 1); ○, protein.
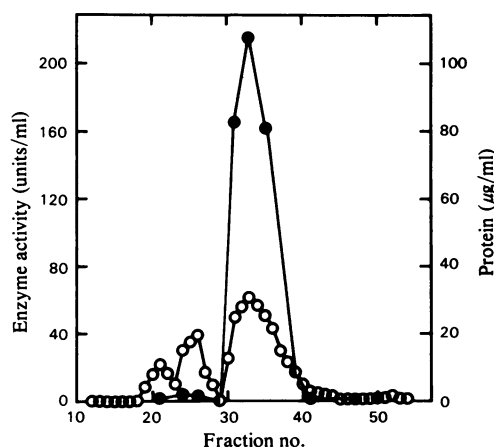


Fig. 3. *Gel filtration of lysyl hydroxylase on a Bio-Gel A-1.5 m column in purification procedure B*
Conditions were as described in the Experimental section. Fractions (1.4 ml) nos. 30–36 were pooled and constituted the purified enzyme. ●, Enzyme activity (for units see Table 1); ○, protein.
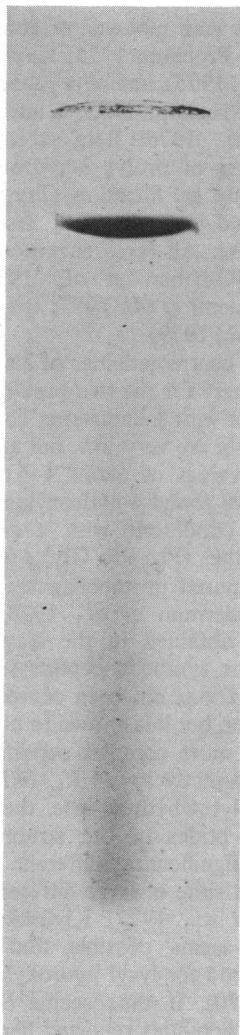
Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis of lysyl hydroxylase purified by procedure B*

Conditions were as described in text. Electrophoretic movement was downward (toward the anode).

column calibrated with protein standards. It has been reported previously that the activity of lysyl hydroxylase purified several hundred- or thousand-fold by conventional steps is found in gel filtration as two peaks with mol.wts. of about 550000 and 200000 (Kivirikko & Prockop, 1972; Ryhänen, 1976). Experiments were therefore carried out to determine whether the other form had been eliminated from the pool during some step in the present purification procedure. The amount of enzyme that did not become bound to concanavalin A–agarose represented less than 5% of the total

lysyl hydroxylase applied to the column in the best experiments, and was hence too small to account for the loss of all the larger form. Furthermore, when this pool was studied by gel filtration, most of the enzyme protein in question was found to correspond in size to the smaller form (results not shown). The enzyme that did not become bound to collagen–agarose and that eliminated from the pool of lysyl hydroxylase eluted from the collagen–agarose (Fig. 2) similarly consisted mainly of the smaller form (results not shown). On the other hand, some preparations which had become inactivated to an appreciable extent during concentration before the final gel filtration had substantial amounts of the activity in an elution position corresponding to the larger form (results not shown). The results thus support previous suggestions that the larger form is not a separate enzyme, but represents an aggregate of the smaller form (Kivirikko & Prockop, 1972; Ryhänen, 1976).

The molecular weight of the enzyme subunit was determined by sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis on 10%(w/v) polyacrylamide gels prepared with 0.5 times the normal amount of cross-linker (Weber & Osborn, 1969). The value obtained for eight separate enzyme preparations was $85000 \pm 2000$ (s.d.), compared with protein standards of known molecular weights (results not shown). Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis with 8% gels gave mol.wts. of 83000–85000 for three preparations.

The synthesis of hydroxylysine by pure lysyl hydroxylase was quantified by using an assay based on the stoichiometric decarboxylation of 2-[1-$^{14}$C]-oxoglutarate during the reaction. The values obtained were corrected to saturated concentrations of the peptide L-I and 2-oxoglutarate by using double-reciprocal plots similar to those shown previously (Puistola *et al.*, 1980a). The concentration of $Fe^{2+}$ was $50 \mu M$ (the apparent $K_m$ being $2-4 \mu M$, see Puistola *et al.*, 1980a), that of $O_2$ $210 \mu M$ (apparent $K_m$ $40-50 \mu M$; Puistola *et al.*, 1980a) and that of ascorbate $1000 \mu M$ (apparent $K_m$ $190-220 \mu M$; Puistola *et al.*, 1980a), the values not being corrected to saturated concentrations in these three cases. Two pure enzyme preparations obtained with procedure B synthesized 46.8 and $76.2 \mu mol$ of hydroxylysine/h per mg of enzyme at 37°C under these conditions. The catalytic-centre activities for these preparations under the conditions given above, as calculated on the basis of a mol.wt. of 200000, are thus 2.6 and 4.2 mol of hydroxylysine/s per mol of enzyme.

## Discussion

Five enzymes, prolyl 4-hydroxylase, prolyl 3-

hydroxylase, lysyl hydroxylase, hydroxylysyl galactosyltransferase and galactosylhydroxylysyl glucosyltransferase, are known to catalyse intracellular post-translational modifications in the collagen domain of procollagen polypeptide chains during their biosynthesis (see Kivirikko & Risteli, 1976; Kivirikko & Myllylä, 1979, 1980; Prockop et al., 1979a,b). Prolyl 4-hydroxylase has been isolated as a homogeneous protein from three sources (see Kivirikko & Myllylä, 1980), and galactosylhydroxylysyl glucosyltransferase from chick embryos (see Kivirikko & Myllylä, 1979). Lysyl hydroxylase thus becomes the third intracellular enzyme of collagen synthesis to be purified to homogeneity.

Previous attempts to isolate lysyl hydroxylase as a homogeneous protein have been hampered by a marked tendency for the enzyme to become inactivated. The two procedures reported here both use only three steps after the initial $(NH_4)_2SO_4$ fractionation. Even then the recoveries of enzyme activity are quite low, in particular in procedure A, mainly owing to this inactivation. By using large amounts of the starting material, however, it is possible to obtain sufficient quantities of pure enzyme for its characterization. It may be noted that, as the activity in the ethylene glycol-containing fractions from the collagen–agarose column in procedure B is very stable, such preparations can be used for experiments requiring highly purified but not pure active enzyme.

The molecular weight of pure lysyl hydroxylase was about 200000 by gel filtration, and that of its monomer about 85000. It thus seems likely that the active enzyme is a dimer consisting of only one type of subunit. As noted in the Results section, the data suggest that the previously reported additional form with an apparent mol.wt. of about 550000 (Kivirikko & Prockop, 1972; Ryhänen, 1976), is not a separate protein, but represents a polymeric form of the enzyme dimer.

Lysyl hydroxylase (Puistola et al., 1980a,b) and prolyl 4-hydroxylase (Myllylä et al., 1977, 1978, 1979; Tuderman et al., 1977) appear to have identical reaction mechanisms, and the kinetic constants for their co-substrates (Myllylä et al., 1977; Tuderman et al., 1977; Puistola et al., 1980a,b) and many other catalytic properties (see Kivirikko & Myllylä, 1980) are likewise very similar. The present data nevertheless indicate that there are distinct differences in the molecular structures of these enzymes. In contrast with the structure for lysyl hydroxylase suggested above, active prolyl 4-hydroxylase is a tetramer with an apparent mol.wt. of about 350000 by gel filtration and a true value of 240000–250000 by sedimentation equilibrium (Pänkäläinen et al., 1970; Berg & Prockop, 1973; Tuderman et al., 1975), and consists of two different

types of subunit with mol.wts. of about 60000 and 64000 (Berg & Prockop, 1973; Kuutti et al., 1975; Tuderman et al., 1975), having a subunit structure of $\alpha_2\beta_2$ (Berg & Prockop, 1973; Kuutti et al., 1975; Tuderman et al., 1975; Berg et al., 1979). The molecular weight of prolyl 3-hydroxylase is only about 160000 by gel filtration (Tryggvason et al., 1977, 1979), and is thus slightly less than that of lysyl hydroxylase. All three enzymes are probably glycoproteins (Guzman et al., 1976; Ryhänen, 1976; Turpeenniemi et al., 1977; Berg et al., 1979; Tryggvason et al., 1979).

The catalytic-centre activities of 2.6 and 4.2mol/s per mol found here for the two pure lysyl hydroxylase preparations with a saturating concentration of L-I as a substrate are very low, but are nevertheless similar to the values of about 4–6mol/s per mol reported for pure prolyl 4-hydroxylase under essentially identical conditions with a saturating concentration of either $(Pro-Pro-Gly)_n$, $n = 5$ or 10, or biologically prepared protocollagen (Berg & Prockop, 1973; Tuderman et al., 1975; Berg et al., 1977). The $V$ obtained in the lysyl hydroxylase reaction with the synthetic peptide substrate of 12 amino acids, L-I, has not been compared with that for protocollagen, but it is known to be identical with that of L-III, a more complex peptide of 33 amino acids with a distinctly lower $K_m$ (Kivirikko et al., 1972). For prolyl 4-hydroxylase, the $V$ values for simple polytripeptides of the structure $(Pro-Pro-Gly)_n$ are not significantly different from that for protocollagen in spite of large differences in the $K_m$ values (Berg et al., 1977; Kivirikko & Myllylä, 1980), and it seems possible that similar conclusions hold good for lysyl hydroxylase (Kivirikko & Myllylä, 1980). It thus seems likely that the catalytic-centre activities reported here are close to the value for the biologically prepared substrate, and that the true catalytic-centre activities for prolyl 4-hydroxylase and lysyl hydroxylase are very similar.

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