Biochem. J. (1988) 253, 489–496 (Printed in Great Britain)

Polyclonal and monoclonal antibodies to human lysyl hydroxylase and studies on the molecular heterogeneity of the enzyme

Raili MYLLYLÄ, Leila PAJUNEN and Kari I. KIVIRIKKO
Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, SF-90220 Oulu, Finland

Human placental lysyl hydroxylase gave two bands in SDS/polyacrylamide-slab-gel electrophoresis: a broad, diffuse, major band corresponding to an apparent M_r of 80000–85000, and a sharp minor band with M_r 78000. Mouse and chick-embryo lysyl hydroxylases gave only the broad, diffuse band, whereas the sharp band could not be detected. Polyclonal antibodies were prepared to the two bands of the human enzyme separately, and monoclonal antibodies were prepared to the whole purified enzyme preparation. Both types of polyclonal antibody inhibited and precipitated the enzyme activity, and both stained the two polypeptide bands in immunoblotting after SDS/polyacrylamide-gel electrophoresis. Only one out of five monoclonal antibodies inhibited the enzyme activity, whereas they all precipitated the activity when studied with antibody coupled to Sepharose. All five monoclonal antibodies stained the whole broad band in immunoblotting, and at least three of them also stained the sharp band. Peptide maps produced from the two polypeptide species by digestion with *Staphylococcus aureus* V8 protease were highly similar. Experiments with endoglycosidase H demonstrated that the M_r-80000–85000 polypeptide contains asparagine-linked carbohydrate units, which are required for maximal lysyl hydroxylase activity. The data suggest that the lysyl hydroxylase dimer consists of only one type of monomer, the heterogeneity of which is due to differences in glycosylation.

INTRODUCTION

Lysyl hydroxylase (EC 1.14.11.4) catalyses the hydroxylation of lysine residues in peptide linkages (for reviews, see Kivirikko & Myllylä, 1980, 1985; Kivirikko & Kuivaniemi, 1986). The reaction occurs as a post-translational event and requires Fe^{2+}, 2-oxoglutarate, molecular O_2 and ascorbate. The hydroxyl group of hydroxylysine residues serves as a site of attachment for carbohydrate units, in the form of either the monosaccharide galactose or the disaccharide glucosylgalactose. Hydroxylysine plays a critical role in the stability of collagens, being essential for the formation of stable intermolecular crosslinks (Eyre, 1980). It is also present in the C1q subcomponent of complement as well as in acetylcholinesterase (see Kivirikko & Myllylä, 1980). The importance of hydroxylysine is clearly demonstrated by the clinical signs of the type VI variant of the Ehlers–Danlos syndrome, a heritable connective-tissue disorder with a deficiency in lysyl hydroxylase activity and marked changes in the mechanical properties of certain tissues (Krane, 1982, 1984; Prockop & Kivirikko, 1984).

Lysyl hydroxylase has been isolated as a homogeneous protein from chick embryos and human placenta (Turpeenniemi-Hujanen *et al.*, 1980, 1981) and in a highly purified form from human foetal tissues (Turpeenniemi-Hujanen *et al.*, 1981). Extensive kinetic studies have been carried out to characterize the reaction which it catalyses and its catalytic properties (Puistola *et al.*, 1980*a*, *b*). The M_r of the enzyme is about 190000 in gel filtration and about 85000 when analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (Turpeenniemi-Hujanen *et al.*, 1980, 1981). Nevertheless, the protein band seen in SDS/polyacrylamide gels is always broad and diffuse, and an additional band corresponding to an M_r of about 78000 is seen in highly purified enzyme preparations from human placenta (see the Results section below). It has therefore not been clear whether the active enzyme consists of one or two types of subunit. Lysyl hydroxylase has a high affinity for concanavalin A–agarose (Turpeenniemi *et al.*, 1977), the binding being decreased in the presence of α-methyl D-mannoside. The enzyme cannot be eluted with this glycoside, however, effective elution being achieved only by a combination of α-methyl D-mannoside and ethylene glycol (Turpeenniemi *et al.*, 1977).

In this present work we prepared polyclonal and monoclonal antibodies to human lysyl hydroxylase for the first time and used these to examine the reasons for the molecular heterogeneity of the enzyme monomer. We also studied whether this molecular heterogeneity might be due to the presence of oligosaccharide units and whether such units have any influence on the catalytic activity of the enzyme.

MATERIALS AND METHODS

Materials

[6-^{3}H]Lysine (17.3 Ci/nmol) was purchased from New England Nuclear; concanavalin A–Sepharose, Protein A–Sepharose CL-4B and Sepharose 4B were from Pharmacia Fine Chemicals; and hydroxyapatite and DEAE-Affi-Gel Blue were from Bio-Rad Laboratories. Protease from *Staphylococcus aureus* V8 was obtained from Boehringer Mannheim, endoglycosidase H from *Streptomyces griseus* from Miles Laboratories, and the Vectastain ABC Kit from Vector Laboratories. [6-^{3}H]-Lysine-labelled protocollagen substrate was prepared in freshly isolated cells from the leg tendons of 17-day chick embryos as described elsewhere (see Kivirikko &

Myllylä, 1982). Collagen–agarose was prepared by coupling citrate-soluble calf skin collagen to Sepharose 4B by the CNBr-activation technique (Kivirikko & Myllylä, 1982).

Purification of lysyl hydroxylase

Lysyl hydroxylase was isolated from human placental tissue, 15-day whole chick embryos and basement-membrane-forming mouse EHS tumours. The purification steps consisted of $(NH_4)_2SO_4$ fractionation, affinity chromatography on concanavalin A–Sepharose, affinity chromatography on collagen linked to agarose and chromatography on hydroxyapatite columns. The present purification procedure is slightly modified from the methods published previously by our laboratory (Turpeenniemi-Hujanen *et al.*, 1980, 1981). The eluate from the collagen–agarose column was diluted by adding 3 vol. of a buffer containing 0.01 M-potassium phosphate, pH 7.5, 0.1 M-NaCl, 0.1 M-glycine and 10 μM-dithiothreitol before application to the hydroxyapatite column equilibrated with the same buffer. After the sample had been applied, the column was washed with a solution containing 0.06 M-potassium phosphate, pH 7.5, 0.1 M-NaCl, 0.1 M-glycine, 10 μM-dithiothreitol and 20% (v/v) ethylene glycol, and the enzyme was eluted with the same solution but containing a linear gradient of potassium phosphate (0.06–0.21 M). To obtain a homogeneous lysyl hydroxylase preparation, the enzyme was re-chromatographed on concanavalin A–Sepharose.

Preparation of polyclonal and monoclonal antibodies to human lysyl hydroxylase

Polyclonal antibodies were produced in rabbits (see Kivirikko & Myllylä, 1987). The two protein bands produced by SDS/polyacrylamide-gel electrophoresis of purified lysyl hydroxylase, with apparent M_r values of about 80000–85000 (LHI) and 78000 (LHII), were excised separately from the slab gels after Coomassie Blue staining and homogenized in 0.14 M-NaCl/0.01 M-sodium phosphate, pH 7.7. Approx. 50 μg of each antigen was emulsified with Freund's complete adjuvant and injected into rabbits intradermally at 10–15 sites. Subsequent boosters of 30 μg were given at 2-week intervals in Freund's incomplete adjuvant.

Monoclonal antibodies were prepared against human lysyl hydroxylase as described by Milstein (Galfre & Milstein, 1981). Balb/c mice were immunized with 50 μg of the purified enzyme three to four times at 3-week intervals, and their spleen lymphocytes were fused with mouse myeloma cells (P3/NS1/1-Ag4-1) in the presence of poly(ethylene glycol), as described previously for prolyl 4-hydroxylase (Höyhtyä *et al.*, 1984). The culture medium of the positive hybridoma cells was used as a starting material for antibody IgG isolation.

Immunoblotting

Partially purified lysyl hydroxylase was electrophoresed on a SDS/8%-polyacrylamide-gel containing 0.5 M-urea in both the buffer and the gel (Weber & Osborn, 1975). The proteins were transferred electrophoretically to a nitrocellulose filter in 40 mM-sodium phosphate buffer (Towbin *et al.*, 1979). The nitrocellulose strips were stained with heparin/toluidine for protein location (Vartio & Vaheri, 1981), and the de-stained strips incubated with immune media or purified IgG in a concentration indicated. The antibodies bound to lysyl hydroxylase were localized with the Vectastain ABC Kit or peroxidase-conjugated anti-IgG (Bio-Rad).

Precipitation of lysyl hydroxylase activity by antibodies coupled to Sepharose

Monoclonal or polyclonal antibody (60 μg) was coupled to Sepharose 4B by the CNBr-activation technique (see Kivirikko & Myllylä, 1982) to prepare an immunoaffinity gel. This gel (0.5 ml) was equilibrated with a buffer containing 0.2 M-NaCl, 0.1 M-glycine, 10% (v/v) ethylene glycol 10 μM-dithiothreitol and 20 mM-Tris/HCl, pH 7.5 at 4 °C. Lysyl hydroxylase (1–5 μg) was added to the gel and incubated at 4 °C for 6 h with gentle agitation. The gel was then separated by centrifugation at 5000 *g* for 10 min and the supernatant assayed for lysyl hydroxylase activity. Control assays were carried out as described above, except that the gel was prepared with the same amount of monoclonal anti-laminin mouse IgG or polyclonal non-immune human IgG coupled to Sepharose 4B.

Cleavage of high-mannose oligosaccharides by endoglycosidase H

Endoglycosidase-H incubation was carried out at 37 °C or at room temperature (21 °C) in 50 mM-sodium citrate buffer, pH 5.0, containing 100 mM-NaCl, 2 mM-phenylmethanesulphonyl fluoride and 0.4 trypsin-inhibitor-unit of aprotinin/ml. The amount of endoglycosidase H in the reactions varied from 0.006 to 0.04 unit/ml and that of lysyl hydroxylase from 15 to 50 μg/ml. When studying the effect of endoglycosidase H on lysyl hydroxylase activity, the digestion was also carried out at pH 7.0.

Peptide maps of protein bands from SDS/polyacrylamide-gel electrophoresis

Purified lysyl hydroxylase was electrophoresed on SDS/polyacrylamide gel. After brief staining with Coomassie Brilliant Blue G, the relevant bands were cut from the gel and subjected to peptide mapping by the procedure of Cleveland *et al.* (1977), with 200 ng of *Staph. aureus* V8 protease per well and digestion for 45 min at room temperature. The gels were stained with silver (see 'Other assays').

Lysyl hydroxylase activity assay

Lysyl hydroxylase activity was assayed by measuring the formation of radioactive hydroxylysine in a [^{3}H]lysine-labelled non-hydroxylated procollagen substrate (see Kivirikko & Myllylä, 1982).

Other assays

Protein bands on SDS/polyacrylamide gels were detected by staining with either Coomassie Brilliant Blue G (Weber & Osborn, 1975) or silver (Wray *et al.*, 1981). A sensitive dansylhydrazine (1-dimethylamino-naphthalene-5-sulphonylhydrazine) method was used for the detection of glycoproteins in SDS/polyacrylamide gels (Eckhardt *et al.*, 1976). The protein content of the enzyme preparations was measured by peptide absorbance at 225 nm, with bovine serum albumin as a standard, which gave an absorption coefficient of $A^{1\ mg/ml} = 7.40$ with a 1 cm light-path.

Purified IgG was prepared from the culture media by either a protein A–Sepharose (MacSween & Eastwood, 1981) or an Affi-Gel Blue (Bruck *et al.*, 1982) affinity

Table 1. Reactivity of lysyl hydroxylase antibodies with the enzyme

Antibody	Type of antibody	Precipitation of activity by antibody coupled to Sepharose* (%)	Inhibition of activity by free antibody† (%)	Antigen binding to immunoblots	
				Diffuse (M_r 80000–85000)	Sharp (M_r 78000)
LHI	Polyclonal	> 90	37	+	+
LHII	Polyclonal	> 90	25	+	+
IF5	Monoclonal	> 90	0	+	−
85B	Monoclonal	> 90	ND	+	−
5F3	Monoclonal	> 90	0	+	+
3F11	Monoclonal	ND	0	+	+
6–3	Monoclonal	> 90	43	+	+

* IgG was coupled to Sepharose 4B and incubated with purified lysyl hydroxylase as described in the Materials and methods section. After incubation, the unbound material was used for activity assay. Equal amounts of monoclonal anti-laminin IgG or polyclonal non-immune IgG in Sepharose 4B behaved identically in the assay and were used as controls. ND, not determined.

† Hybridoma medium or immune serum was used in the activity assay. Activity was compared with that obtained in the presence of the same amount of culture medium or non-immune serum, respectively. Values represent the maximal inhibition (Fig. 1) obtained in the presence of about 1 μg of lysyl hydroxylase and up to 150 μl of immune medium or serum. Similar results are obtained with antibody IgG.

column, with 0.1 M-glycine/HCl, pH 3, or 50 mM-NaCl/20 mM-Tris/HCl, pH 7.2, at 4 °C for elution of the antibodies.

RESULTS

Polyclonal and monoclonal antibodies and their use for characterizing human lysyl hydroxylase

Human placental lysyl hydroxylase gave two main bands in SDS/polyacrylamide-slab-gel electrophoresis after reduction and denaturation of the polypeptide chains: a broad diffuse band with an apparent M_r of 80000–85000, and a sharp band with an apparent M_r of 78000 (see Fig. 3, lane 2). The latter band was not seen, however, if the enzyme preparation was re-chromatographed on concanavalin A–Sepharose (results not shown).

Polyclonal antibodies to these protein bands were prepared separately by using the corresponding bands excised from the slab gels as the antigens. Monoclonal antibodies were produced to enzyme preparations containing both major bands. Two sets of fusions were performed by using two mice with positive serum titres. Initial screening of hybridoma culture supernatants revealed that 22 culture supernatants out of 960 wells were positive by enzyme-linked immunosorbent assay (Höyhtyä *et al.*, 1984). Five hybridoma clones, all derived from different primary wells, were chosen for further characterization.

The two polyclonal antibodies both inhibited the activity of human placental lysyl hydroxylase when added to the enzyme-activity incubation mixture, but complete inhibition was not achieved (Table 1 and Fig. 1). Both antibodies also precipitated the enzyme activity when examined with antibodies coupled to Sepharose, the maximal degree of precipitation obtained approaching 100% (Table 1). Only one of the monoclonal antibodies partially inhibited the enzyme activity (Table 1 and Fig. 1), whereas all antibodies tested precipitated this activity in experiments with IgG coupled to Sepharose (Table 1).

Both polyclonal antibodies, i.e. those prepared separately for the M_r 80000–85000 protein band and the M_r-78000 band, stained both polypeptide species in immunoblotting after SDS/polyacrylamide-slab-gel electrophoresis (Table 1, Fig. 2*a*). All monoclonal antibodies stained the M_r-80000–85000 band (Table 1). Although the staining was very weak throughout, it could be clearly seen that the whole broad diffuse band was immunostained (Fig. 2*b*). At least three of the five monoclonal antibodies also stained the M_r-78000 band, whereas with two of these antibodies such staining could not be detected. None of the monoclonal antibodies

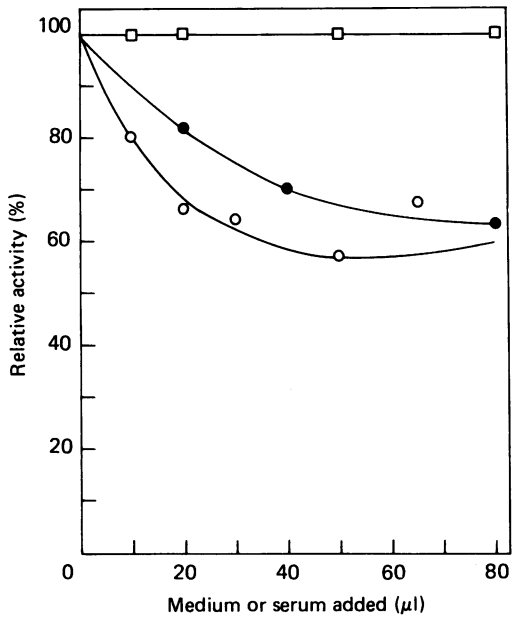

Fig. 1. Effect of antibodies on lysyl hydroxylase activity

Different amounts of hybridoma medium or immune serum were added to the lysyl hydroxylase reaction containing about 1 μg of the enzyme. The activity values are expressed as percentage of values obtained in the presence of the same amount of culture medium or non-immune serum, respectively. ●, LHI immune serum; ○, 6–3 immune medium; □, 5F3 immune medium.

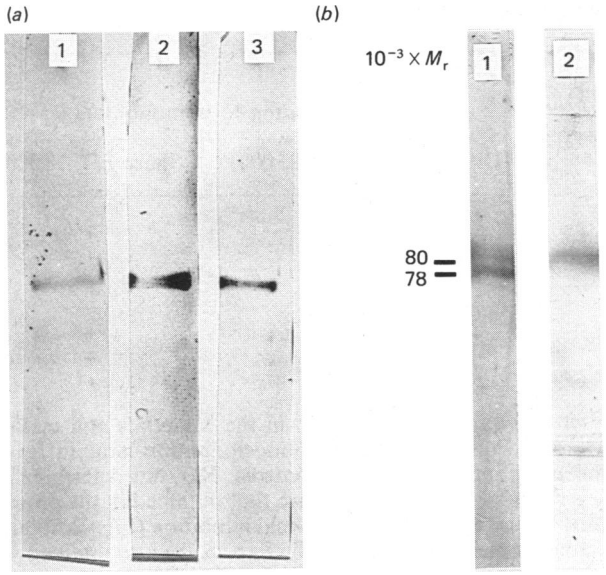

Fig. 2. Immunoblotting of highly purified lysyl hydroxylase from human placenta

(*a*) Purified lysyl hydroxylase was run on SDS/polyacrylamide-gel electrophoresis, the protein band of apparent M_r 80000–85000 was excised from the gel and re-run, after which it was blotted on a nitrocellulose filter and immunostained. Lane 1, heparin/toluidine staining; lane 2, immunostaining with polyclonal antibody LHI (IgG concn. 10 μg/ml); lane 3, immunostaining with polyclonal antibody LHII (IgG concn. 20 μg/ml). (*b*) Purified lysyl hydroxylase was run on SDS/polyacrylamide-gel electrophoresis, blotted on a nitrocellulose filter and then immunostained with immune media. Lane 1, results obtained with a monoclonal antibody (6–3) that stains both the M_r-80000–85000 and M_r-78000 band; lane 2, results with an antibody (1F5) that gave no detectable staining of the lower-M_r band.

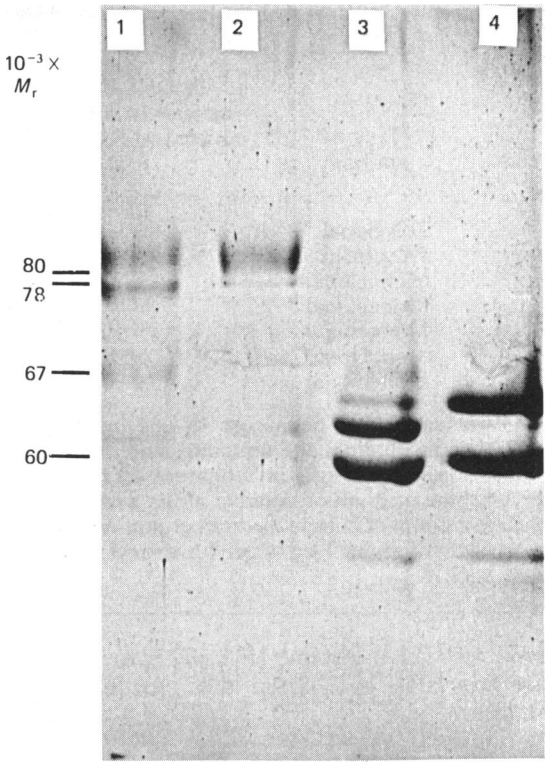

Fig. 3. Effect of endoglycosidase H on highly purified human placental lysyl hydroxylase

Lysyl hydroxylase was isolated from human placental tissue by the purification procedure described in the Materials and methods section, but omitting the re-chromatography on concanavalin A–Sepharose. The purified enzyme was electrophoresed on SDS/polyacrylamide gel, and stained with Coomassie Brilliant Blue. Lane 1, highly purified lysyl hydroxylase after endoglycosidase H treatment (see the Materials and methods section); lane 2, highly purified lysyl hydroxylase without endoglycosidase H treatment. Purified prolyl 4-hydroxylase (see Kivirikko & Myllylä, 1982) from chick embryos with (lane 3) and without (lane 4) endoglycosidase H treatment was used for comparison.

stained the M_r-78000 band alone without recognizing the M_r-80000–85000 band (Table 1).

Treatment of human lysyl hydroxylase by endoglycosidase H

Endoglycosidase H cleaves the glycoside bond between two *N*-acetylglucosamine residues of asparagine-linked oligosaccharides (Tarentino & Maley, 1974), resulting in the removal of high-mannose oligosaccharide units. Incubation of purified human placental lysyl hydroxylase with endoglycosidase H resulted in a distinct decrease in the apparent M_r of the M_r-80000–85000 protein band (Fig. 3). The M_r of the lysyl hydroxylase polypeptide was 78000 after removal of the oligosaccharide units, and thus the apparent carbohydrate contribution to the M_r in SDS/polyacrylamide-gel electrophoresis was about 5000. This new protein band appeared at the same location as the sharp band present in highly purified enzyme preparations from human placenta (Fig. 3). The sharp band (M_r 78000) became more prominent after endoglycosidase H treatment, whereas the diffuse band of the active enzyme decreased in intensity (see also Fig. 4). The new M_r-67000 polypeptide is endoglycosidase H.

Heterogeneity of mouse and chick-embryo lysyl hydroxylase and effect of endoglycosidase H

A slight difference was found in apparent M_r between the human, mouse and chicken lysyl hydroxylase (Fig. 4). This was demonstrated by immunostaining (results not shown) of the broad protein band in the chicken samples of M_r 81000–88000 and the markedly broader band (M_r 81000–91000) in the mouse samples with a polyclonal anti-(human lysyl hydroxylase) antibody (LHI in Table 1). The mouse and chick enzyme preparations did not contain any detectable amounts of a protein band corresponding to the human M_r-78000 polypeptide. Treatment of the mouse and chick lysyl hydroxylases with endoglycosidase H decreased the M_r of the enzyme proteins. After the removal of the asparagine-linked oligosaccharide units, the M_r values of the chicken and mouse lysyl hydroxylases were identical, but slightly higher than that of the human lysyl hydroxylase, corresponding to an M_r of about 80000 and 78000

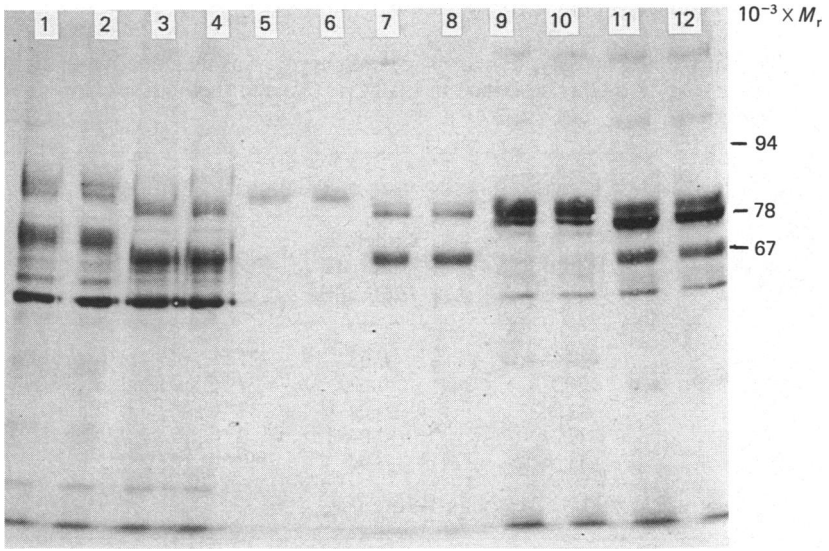

Fig. 4. Effect of endoglycosidase H on mouse and chick-embryo lysyl hydroxylase

Lysyl hydroxylase from a mouse EHS tumour (lanes 1–4), chick embryos (lanes 5–8) and human placenta (lanes 9–12) was purified as described in the Materials and methods section. The mouse enzyme was purified by concanavalin A–Sepharose and a collagen–agarose column, whereas the enzymes from the chick embryos and human placenta were chromatographed, in addition to the above columns, on a hydroxyapatite column. Proteins were separated by SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and detected by heparin/toluidine staining. Lanes 3 and 4 show mouse lysyl hydroxylase, lanes 7 and 8 chick-embryo lysyl hydroxylase and lanes 11 and 12 human placental lysyl hydroxylase after treatment by endoglycosidase H. The protein band in these lanes corresponding to M_r 67000 is endoglycosidase H. Because of only partial purification, the enzyme preparations contain other proteins present as contaminants. Positions of M_r markers are shown.

respectively. The data suggest that asparagine-linked oligosaccharides contribute similarly to the apparent M_r of the enzyme protein in different species, and thus these oligosaccharides alone do not account for the M_r differences.

Peptide mapping of human lysyl hydroxylase

Peptide mapping was performed to clarify further the nature of the two lysyl hydroxylase polypeptides. The M_r-78000 protein band and the similar-sized protein band after endoglycosidase H treatment of purified lysyl hydroxylase were excised from the SDS/polyacrylamide gels and digested with *Staph. aureus* V8 protease. The two proteins were found to be extremely similar, if not identical (Fig. 5), suggesting that the M_r-78000 protein is an under-glycosylated or non-glycosylated form of lysyl hydroxylase.

After endoglycosidase H treatment, a small amount of protein usually remained in the diffuse band area of lysyl hydroxylase in the SDS/polyacrylamide gel. The V8-protease map of this residual protein was very similar to that of the M_r-78000 protein obtained from lysyl hydroxylase by the endoglycosidase H treatment, although the presence of minor differences cannot be excluded (Fig. 5). This result indicates that the cleavage by endoglycosidase H was incomplete under conditions that produce a complete digestion of much larger amounts of prolyl 4-hydroxylase (cf. Fig. 3). The reason for this difference between the two hydroxylases is unknown. The data from the V8-protease and antibody studies suggest that human lysyl hyroxylase contains only one polypeptide chain type.

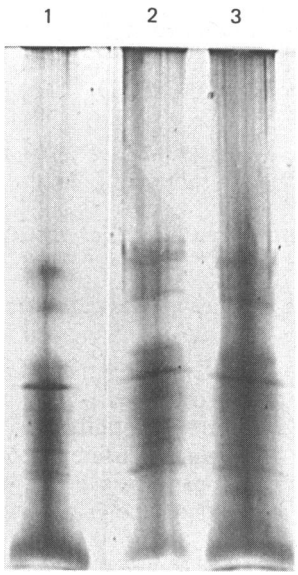

Fig. 5. V8-protease digestion of highly purified human placental lysyl hydroxylase

The protein band remaining from lysyl hydroxylase after endoglycosidase H treatment, corresponding to M_r 80000–85000 (lane 1), that appearing from lysyl hydroxylase after endoglycosidase H treatment, corresponding to M_r 78000 (lane 2), and that present in highly purified lysyl hydroxylase from human placenta, corresponding to M_r 78000 (lane 3), were digested with V8 protease (see the Materials and methods section). The peptides were separated by SDS/polyacrylamide-gel electrophoresis and detected by silver staining.

Table 2. Effect of treatment with endoglycosidase H on lysyl hydroxylase activity

For determination of lysyl hydroxylase activity, human placental enzyme was incubated with endoglycosidase H (Endo H) or with the buffer lacking endoglycosidase H as described in the Materials and methods section, after which part of the solution was used to assay the enzyme activity.

Expt. no.	Lysyl hydroxylase activity (d.p.m.)		Treatment conditions		Inhibition of lysyl hydroxylase activity (%)
	With Endo H	Without Endo H	Concn. of Endo H (unit/ml)	Temperature, pH, incubation time	
1	708	1148	0.02	21 °C, pH 7.0, overnight	38
2	760	2087	0.03	21 °C, pH 5.0, overnight	64
3	521	1124	0.04	37 °C, pH 7.0, 2 h	54
4	1087	1124	0.04	4 °C, pH 7.0, 2 h	3
5	1252*	1124	0.04	37 °C, pH 7.0, 2 h	0

* Endoglycosidase H was denatured at 90 °C for 5 min before use.

Table 3. Catalytic activity of the underglycosylated form of lysyl hydroxylase

Lysyl hydroxylase was treated with endoglycosidase H (see the Materials and methods section). Equal volumes of a solution containing 10 mM-$MnCl_2$, 0.2 M-NaCl, 0.1 M-glycine and 0.02 M-Tris HCl, pH 7.4 at 4 °C, were added, and the samples were mixed at 4 °C for 1 h with 0.1 ml of concanavalin A–Sepharose or Sepharose 4B. After centrifugation at 2000 *g* for 10 min, the supernatant was assayed for lysyl hydroxylase activity.

Chromatography on	Treatment with endoglycosidase H	Lysyl hydroxylase activity		
		In column supernatant (d.p.m.)	Bound to column (d.p.m.)	Bound to column (%)
Concanavalin A–Sepharose	−	300	15090†	98
Sepharose 4B	−	15390*	0	0
Concanavalin A–Sepharose	+	4740	7875†	51
Sepharose 4B	+	12615*	0	0

* Total activity was 12615 d.p.m. after endoglycosidase H treatment, and 15390 d.p.m. in the untreated samples.
† Value calculated by subtracting d.p.m. not bound to concanavalin A–Sepharose from d.p.m. measured in Sepharose 4B supernatant.

Effect of oligosaccharides on the catalytic activity of lysyl hydroxylase

In order to examine the importance of oligosaccharide units for lysyl hydroxylase activity, the oligosaccharide chains were removed from partially purified enzyme by treatment with endoglycosidase H. Many separate experiments with endoglycosidase H were performed under different conditions, and a distinct inhibition of the enzyme activity was obtained (Table 2). Additional bovine serum albumin (1 mg/ml) was added to the incubation solution containing endoglycosidase H to ensure that the inhibition of the enzyme was not due to any contaminating proteinases present in the endoglycosidase H preparations. No difference was observed between the two types of experiments, however (results not shown), and no signs of protein degradation after the treatment were seen by SDS/polyacrylamide-gel electrophoresis. In all cases inhibition was only partial, no total inhibition being observed after endoglycosidase H treatment. Inhibition varied from about 10 to 60% in different experiments.

After endoglycosidase H treatment, the lysyl hydroxylase was chromatographed on concanavalin A–Sepharose to separate the glycosylated and non-glycosylated forms. As shown in Table 3, native lysyl hydroxylase was bound quantitatively to concanavalin A–Sepharose, whereas after endoglycosidase H treatment only 51% of the original lysyl hydroxylase activity became bound to the column. Activity was recovered in the unbound material, but this non-glycosylated form showed only 31% of the expected 49% of the original activity. From these calculations, the non-glycosylated form appears to possess 63% of the activity found in glycosylated lysyl hydroxylase. Three additional experiments gave values of 40%, 62% and 63% for the non-glycosylated lysyl hydroxylase, values which correlate well with the maximum inhibition found after endoglycosidase H treatment in Table 2.

DISCUSSION

Lysyl hydroxylase from human placental tissue, mouse EHS tumour and chick embryos was found to be heterogeneous when studied by SDS/polyacrylamide-gel

electrophoresis after reduction and denaturation of the polypeptide chains. The human enzyme consisted of a broad, diffuse, M_r-80000–85000 major band and a sharp M_r-78000 minor band, whereas the latter band could not be detected in the mouse and chick enzyme preparations. Polyclonal antibodies prepared to either of the two bands of the human enzyme precipitated the enzyme activity and stained both polypeptide species in immunoblotting after SDS/polyacrylamide-gel electrophoresis. Most of the monoclonal antibodies also detected both polypeptide species. The two polypeptides were further very similar in peptide maps produced after digestion with *Staph. aureus* V8 protease, and no evidence was found for the existence of an additional polypeptide remaining in the diffuse-band region after treatment with endoglycosidase H. The data thus suggest that lysyl hydroxylase consists of only one type of polypeptide chain. Because the M_r of the enzyme by gel filtration (Turpeenniemi-Hujanen *et al.*, 1980, 1981) is about twice the value found here, the active lysyl hydroxylase is probably a dimer.

The molecular heterogeneity of the lysyl hydroxylase monomer was found to be due to heterogeneity in its glycosylation. The data obtained in the experiments with endoglycosidase H, together with previous evidence from lectin affinity chromatography (Turppeenniemi *et al.*, 1977), show that lysyl hydroxylase is a glycoprotein containing mannosyl residues linked to one or more asparagine residues in the enzyme polypeptide chain via *N*-acetylglucosamine. These oligosaccharide units increase the apparent M_r of the enzyme subunit by about 5000. The increase was similar in human, mouse and chicken lysyl hydroxylases, although slight differences in the apparent M_r values of lysyl hydroxylase were found between these sources. The glycoprotein character of lysyl hydroxylase was confirmed by dansylhydrazine, a sensitive glycoprotein-staining agent, which gave a brilliant staining of the broad band of lysyl hydroxylase in SDS/polyacrylamide-slab-gel electrophoresis (results not shown). After removal of the oligosaccharides, the broad, diffuse, M_r-80000–85000 band of lysyl hydroxylase became sharper, suggesting that the diffuse migration, which was an especially prominent feature in mouse EHS tumour lysyl hydroxylase, was due to microheterogeneity in the carbohydrate side chains. It should be noted that a highly purified preparation of lysyl hydroxylase from human placenta also contained a non-glycosylated form of the polypeptide chain which was not detectable in the chicken and mouse tissues. Whether the non-glycosylated form is also present in the placenta *in vivo* or whether it is formed during the purification procedure remains to be determined.

Incubation of lysyl hydroxylase with endoglycosidase H decreased the enzyme activity and gave an underglycosylated form of lysyl hydroxylase, which was found to have 40–60 % of the catalytic activity measured in the glycosylated enzyme. It thus seems likely that asparagine-linked oligosaccharides are required to obtain maximum lysyl hydroxylase activity.

Lysyl hydroxylase is very similar to prolyl 4-hydroxylase in its catalytic properties (Kivirikko & Myllylä, 1980, 1985). Lysyl hydroxylase differs distinctly from prolyl 4-hydroxylase, however, consisting of only one type of monomer, whereas prolyl 4-hydroxylase is an $\alpha_2\beta_2$ tetramer (Kivirikko & Myllylä, 1980, 1985). In prolyl 4-hydroxylase the α-subunit contributes a major part of the catalytic site of the enzyme, as it probably contains the peptide-binding region (de Waal *et al.*, 1985) and the 2-oxoglutarate-attachment site (Günzler *et al.*, 1987; de Waal *et al.*, 1987), whereas both the α-subunit and β-subunit appear to contribute to the ascorbate-binding site (Günzler *et al.*, 1988). Thus in lysyl hydroxylase the single enzyme monomer must also contain amino acid sequences of the kind that contribute to the active site of prolyl 4-hydroxylase in the β-subunit.

Polyclonal and monoclonal antibodies to human lysyl hydroxylase were prepared for the first time in the course of the present study. Only one of the monoclonal antibodies had a weak inhibitory effect on lysyl hydroxylase activity, suggesting that most of them were not reacting at the active site of the enzyme. The different polyclonal and monoclonal antibodies prepared here may provide useful tools for examining lysyl hydroxylase behaviour in patients with type VI of the Ehlers–Danlos syndrome. Furthermore, these antibodies give an opportunity to screen lysyl hydroxylase recombinants from expression libraries, marking the initiation of research into lysyl hydroxylase at the cDNA and genomic DNA level.

We gratefully acknowledge the expert technical assistance of Mrs. Sirkka Vilmi.

REFERENCES

Bruck, C., Portetelle, D., Glineur, C. & Bollen, A. (1982) J. Immunol. Methods **53**, 313–319

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) J. Biol. Chem. **252**, 1102–1106

de Waal, A., de Jong, L., Hartog, A. F. & Kemp, A. (1985) Biochemistry **24**, 6493–6499

de Waal, A., Hartog, A. F. & de Jong, L. (1987) Biochim. Biophys. Acta **912**, 151–155

Eckhardt, A. E., Hayes, C. E. & Goldstein, I. J. (1976) Anal. Biochem. **73**, 192–197

Eyre, D. R. (1980) Science **207**, 1315–1322

Galfre, G. & Milstein, C. (1981) Methods Enzymol. **73B**, 3–46

Günzler, V., Hanauske-Abel, H. M., Myllylä, R., Mohr, J. & Kivirikko, K. I. (1987) Biochem. J. **242**, 163–169

Günzler, V., Hanauske-Abel, H. M., Myllylä, R., Kaska, D., Hanauske, A. & Kivirikko, K. I. (1988) Biochem. J. **251**, 365–372

Höyhtyä, M., Myllylä, R., Piuva, J., Kivirikko, K. I. & Tryggvason, K. (1984) Eur. J. Biochem. **141**, 477–482

Kivirikko, K. I. & Kuivaniemi, H. (1986) in Connective Tissue Disease: Molecular Pathology of the Extracellular Matrix (Uitto, J. & Perejda, A. J., eds.), pp. 263–292, Marcel Dekker, New York

Kivirikko, K. I. & Myllylä, R. (1980) in The Enzymology of Post-Translational Modification of Proteins (Freedman, R. B. & Hawkins, H. C., eds.), pp. 53–104, Academic Press, New York

Kivirikko, K. I. & Myllylä, R. (1982) Methods Enzymol. **82**, 245–304

Kivirikko, K. I. & Myllylä, R. (1985) Ann. N.Y. Acad. Sci. **460**, 187–201

Kivirikko, K. I. & Myllylä, R. (1987) Methods Enzymol. **144**, 96–114

Krane, S. M. (1982) in American Academy of Orthopaedic Surgeons Symposium on Heritable Disorders of Connective Tissue (Akeson, W. H., Bornstein, P. & Glimcher, M. J., eds.), pp. 61–75, Mosby, St. Louis

Krane, S. M. (1984) in Extracellular Matrix Biochemistry (Piez, K. A. & Reddi, A. H., eds.), pp. 413–463, Elsevier, New York
MacSween, J. M. & Eastwood, S. L. (1981) Methods Enzymol. **73B**, 459–471
Prockop, D. J. & Kivirikko, K. I. (1984) N. Engl. J. Med. **311**, 376–386
Puistola, U., Turpeenniemi-Hujanen, T. M., Myllylä, R. & Kivirikko, K. I. (1980*a*) Biochim. Biophys. Acta **611**, 40–50
Puistola, U., Turpeenniemi-Hujanen, T. M., Myllylä, R. & Kivirikko, K. I. (1980*b*) Biochim. Biophys. Acta **611**, 51–60
Tarentino, A. L. & Maley, F. (1974) J. Biol. Chem. **249**, 811–817
Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
Turpeenniemi, T. M., Puistola, U., Anttinen, H. & Kivirikko, K. I. (1977) Biochim. Biophys. Acta **483**, 215–219
Turpeenniemi-Hujanen, T. M., Puistola, U. & Kivirikko, K. I. (1980) Biochim. J. **189**, 247–253
Turpeenniemi-Hujanen, T. M., Puistola, U. & Kivirikko, K. I. (1981) Collagen Relat. Res. **1**, 355–366
Vartio, T. & Vaheri, A. (1981) J. Biol. Chem. **256**, 13085–13090
Weber, K. & Osborn, M. (1975) in The Proteins (Neurath, H. & Hill, R. L., eds.), vol. 1, pp. 179–223, Academic Press, New York
Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981) Anal. Biochem. **118**, 197–203

Received 9 November 1987/2 March 1988; accepted 31 March 1988