Catalytic properties of lysyl hydroxylase from cells synthesizing genetically different collagen types

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Crude preparations of lysyl hydroxylase were extracted from chick-embryo tendons synthesizing exclusively type I collagen, chick-embryo sterna synthesizing exclusively type II collagen and HT-1080 sarcoma cells synthesizing exclusively type IV collagen. No differences were found in the K_m values for Fe^{2+} , 2-oxoglutarate and ascorbate between these three enzyme preparations. Similarly no differences were found in the K_m values for type I and type II protocollagens and the rate at which type IV protocollagen is hydroxylated between these enzyme preparations. The extent to which type I protocollagen could be hydroxylated by the three enzymes was likewise identical. These data strongly argue against the existence of collagen-type-specific lysyl hydroxylase isoenzymes.

Lysyl hydroxylase (peptidyl-lysine, 2-oxoglutarate: oxygen 5-oxidoreductase, EC 1.14.11.4) catalyses the synthesis of hydroxylysine in collagen by the hydroxylation of lysyl residues in peptidyl linkages. The reaction occurs as a post-translational modification and requires 2-oxoglutarate, molecular oxygen, Fe^{2+} ions and ascorbate. The hydroxy groups of the hydroxylysyl residues serve as the sites of attachment for carbohydrate units and they are important in stabilizing the intramolecular collagen cross-links (for recent review, see Kivirikko & Myllylä, 1980).

The extent of lysyl hydroxylation varies in different collagen types and within the same type from different tissues (Kivirikko & Myllylä, 1980), as also does the lysyl hydroxylation defect encountered in the type VI variant of the Ehlers–Danlos syndrome (Pinnell *et al.*, 1972; Krane, 1980). One reason for these differences might be the existence of collagen-type-specific or tissue-specific lysyl hydroxylase isoenzymes.

On the other hand, lysyl hydroxylases purified to homogeneity from chick embryos and human placental tissues and to essential homogeneity from human foetal tissues (Turpeenniemi-Hujanen *et al.*, 1980, 1981) have an identical molecular weight for the subunit and identical kinetic constants for the co-substrates when determined with type I protocollagen as the substrate (Puistola *et al.*, 1980; Turpeenniemi-Hujanen *et al.*, 1981). Additionally, no significant immunological differences have been found between the lysyl hydroxylases from whole chick embryo, chick-embryo tendon, cartilage and

kidneys in double immunodiffusion and antiserum-inhibition studies (Turpeenniemi-Hujanen, 1981).

The present work studies the catalytic properties of lysyl hydroxylases from chick-embryo tendons synthesizing exclusively type I collagen (see Bornstein & Sage, 1980), chick-embryo sterna synthesizing exclusively type II collagen (Bornstein & Sage, 1980) and human HT-1080 sarcoma cells synthesizing exclusively type IV collagen (Alitalo *et al.*, 1980; Pihlajaniemi *et al.*, 1981). The object of these experiments was to elucidate further the possible existence of collagen type-specific lysyl hydroxylase isoenzymes.

Experimental

Materials

DL-[6- $^3\text{H}(\text{n})$]Lysine was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.) and HT-1080 sarcoma cells (ATCC CLL 121) were from the American Type Culture Collection, Rockville, MD, U.S.A. Fertilized eggs of white Leghorn chickens were obtained from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and were incubated at 37°C in a moist atmosphere until used.

Extraction of lysyl hydroxylase

Lysyl hydroxylase was extracted from the tendons or sterna of 15-day chick embryos by homogenization in a solution containing 0.1 M-glycine, 0.2 M-NaCl, 0.1% (w/v) Triton X-100, 0.01% (w/v) soya-bean trypsin inhibitor and 0.02 M-Tris/HCl buffer (pH 7.6 at 4°C) (Risteli *et al.*, 1979) for

5 × 5 s with an Ultra-Turrax homogenizer. The homogenates were centrifuged at 15000 *g* for 30 min at 4°C and the supernatants used as the 'type I' and 'type II' enzymes.

The lysyl hydroxylase referred to as the 'type IV' enzyme was isolated from HT-1080 sarcoma cells grown in 75 cm² plastic tissue-culture bottles in a total volume of 20 ml at 37°C in a humidified incubator with an atmosphere of air/CO₂ (19:1). The medium was Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) newborn-calf serum, 50 µg of ascorbic acid/ml added daily, 50 units of penicillin/ml and 50 µg of streptomycin/ml. The cells were harvested by trypsin treatment in Krebs medium. The cell pellet was isolated by centrifugation at 600 *g* for 3 min and washed three times with 10% newborn-calf serum in Krebs solution. The cells were homogenized in the homogenization buffer described above with a Teflon/glass homogenizer and centrifuged at 15000 *g* for 30 min at 4°C. The supernatant was used as the enzyme.

Preparation of protocollagen substrates

The protocollagen substrates were prepared from 16-day chick-embryo tendon cells, 15-day chick-embryo sterna cells and cultured HT-1080 sarcoma cells according to the principles described previously (Risteli & Kivirikko, 1976), except that DL-[6-³H(n)]lysine was used instead of [¹⁴C]lysine. The leg tendons or sterna of the chick embryos were dissected and the cells isolated by controlled digestion with trypsin and purified bacterial collagenase (Dehm & Prockop, 1972) and then filtered through lens paper. The liberated tendon and sternum cells, and also the HT-1080 sarcoma cells, which were harvested with trypsin, were washed three times with modified Krebs medium containing 10% newborn-calf serum (Dehm & Prockop, 1972; Berg & Prockop, 1973).

The cells were incubated at 37°C in modified Krebs medium (Dehm & Prockop, 1972) containing 5% newborn-calf serum, preincubated with αα'-bipyridine for 25 min and pulsed with 500 µCi of

DL-[6-³H(n)]lysine for 3 h for the tendon cells, 1.5 h for the sternum cells and 2 h for the HT-1080 sarcoma cells. The cells were homogenized and the protocollagen synthesized was partially purified by acetic acid extraction (Berg & Prockop, 1973). These protocollagen substrates are referred to as 'type I', 'type II' and 'type IV' substrates respectively. The specific radioactivity of the free lysine pool was measured and that of each substrate preparation calculated as described by Breul *et al.* (1980).

Assay of lysyl hydroxylase activity

Lysyl hydroxylase activity was assayed by incubating the 15000 *g* supernatants with different DL-[6-³H(n)]lysine-labelled protocollagen substrates for 30 min at 37°C in a final volume of 1 ml containing 0.05 mM-FeSO₄, 0.5 mM-2-oxoglutarate, 1.0 mM-ascorbic acid, 0.1 mg of catalase (Calbiochem), 0.1 mM-dithiothreitol (Calbiochem), 1.5 mg of bovine serum albumin (Sigma) and 50 mM-Tris/HCl buffer adjusted to pH 7.8 at 25°C. The reaction was carried out as described previously (Kivirikko & Prockop, 1972; Puistola *et al.*, 1980).

Results

Kinetic constants of co-substrates for the lysyl hydroxylases from chick-embryo tendons and sterna and HT-1080 sarcoma cells

The *K_m* values for ascorbate, 2-oxoglutarate and Fe²⁺ ions were determined by varying the protocollagen-type-I substrate concentration at different fixed concentrations of ascorbate, 2-oxoglutarate or Fe²⁺ and constant concentrations of the other components. The analyses were performed with the 'type I', 'type II' and 'type IV' enzyme preparations, and the apparent *K_m* values were calculated from the secondary plots (Plowman, 1972; Puistola *et al.*, 1980). The *K_m* values obtained for the three enzyme preparations were identical within the limits of experimental error (Table 1).

Table 1. Apparent kinetic constants for the co-substrates of 'type I', 'II' and 'IV' lysyl hydroxylases
The kinetic constants were determined from secondary transforms of the primary plots as described in the text. The substrate was DL-[6-³H(n)]lysine-labelled protocollagen from chick-embryo tendon cells.

Enzyme source	<i>K_m</i> (µM)		
	2-Oxoglutarate	Ascorbate	Fe ²⁺
Chick-embryo tendon (type I)	70	220	2.0
Chick-embryo sternum (type II)	80	260	1.0
HT-1080 sarcoma cells (type IV)	70	210	1.5

Fig. 1. K_m values for type-I procollagen substrate with lysyl hydroxylase from chick-embryo tendons (a) and human HT-1080 sarcoma cells (b)

The concentration of the type-I procollagen substrate was varied at different fixed concentrations of 2-oxoglutarate (●, 12.5 μM ; ○, 25 μM ; ■, 125 μM). The maximal-velocity values cannot be compared from one experiment to another. The velocity (v) is expressed as d.p.m. of hydroxylysine formed in 30 min.

Comparison of lysyl hydroxylation with type-I, -II and -IV procollagen substrates

The K_m values for the type-I and -II procollagen substrates were determined by varying the procollagen substrate concentration at different fixed concentrations of 2-oxoglutarate and constant concentrations of the other reactants (Figs. 1 and 2). The apparent K_m values were determined

Fig. 2. K_m values for type-II procollagen substrate with lysyl hydroxylase from chick-embryo sterna (a) and human HT-1080 sarcoma cells (b)

The concentration of the type-II procollagen substrate was varied at different fixed concentrations of 2-oxoglutarate (●, 12.5 μM ; ○, 25 μM ; ■, 125 μM). The maximal-velocity values cannot be compared from one experiment to another. The velocity (v) is expressed as d.p.m. of hydroxylysine formed in 30 min.

from the data obtained in initial-velocity studies and calculated from the primary plots (Plowman, 1972; Puistola *et al.*, 1980). No differences were seen between the K_m values in respect of the type-I or type-II procollagen substrates for any of the three enzyme preparations (Table 2).

Owing to technical difficulties encountered in preparing large amounts of type IV procollagen, K_m values were not determined for this substrate. However, when the same amounts of activity units of the 'type I', 'II' and 'IV' enzymes were taken, as tested with type-I procollagen substrate, and these samples were assayed with type-IV procollagen substrate, identical amounts of the activity units

Table 2. Apparent K_m values for type-I and type-II procollagen substrate with 'type I', 'II' and 'IV' lysyl hydroxylases. The apparent K_m values were determined from data obtained in initial-velocity studies and were calculated from primary plots. The substrate was DL-[6- 3 H(n)]lysine-labelled procollagen from chick-embryo tendon or sternum cells.

Enzyme source	Protocollagen ...	K_m (nM)	
		Type I	Type II
Chick-embryo tendon ('type I')		5-7	6
Chick-embryo sternum ('type II')		7-10	8
HT-1080 sarcoma cell ('type IV')		7-10	9

Fig. 3. Hydroxylation of type I procollagen with 'type I', 'II' and 'IV' lysyl hydroxylases

DL-[6- 3 H(n)]Lysine-labelled procollagen type-I substrate from chick-embryo tendon cells (3.4 nmol) was hydroxylated with lysyl hydroxylase from chick-embryo tendons (type I) (●), chick-embryo sternum (type II) (△) and HT-1080 sarcoma cells (type IV) (○). Lysyl hydroxylation was measured as d.p.m./30 min, one unit of enzyme activity being defined as the amount of enzyme present in 1 mg of the $(\text{NH}_4)_2\text{SO}_4$ fraction (17-55% saturation) obtained from the 15000g supernatant of chick-embryo homogenate (Kivirikko & Prockop, 1972; Turpeenniemi-Hujanen *et al.*, 1980).

were again found in these three enzyme preparations (Table 3). Similarly, when the same amounts of type-I procollagen substrate were hydroxylated with the 'type I', 'II' and 'IV' enzymes, no differences were seen in the extent to which the type I procollagen could be hydroxylated (Fig. 3).

Discussion

The present data indicate that the K_m values for type I and type II procollagens and the rate at which type IV procollagen is hydroxylated are identical for crude lysyl hydroxylase preparations from cells synthesizing exclusively type I, II or IV collagen. The extent to which type I procollagen can be hydroxylated with lysyl hydroxylase from these three sources was likewise found to be identical. Additional similarity in the catalytic properties of these three enzyme preparations was found in that the K_m values for Fe^{2+} , 2-oxoglutarate and ascorbate were identical within the limits of experimental error. The K_m values determined here for the co-factors and co-substrates and with respect to the type-I procollagen substrate are also very similar to those previously determined for purified or pure lysyl hydroxylase from chick embryos (Ryhänen & Kivirikko, 1974; Puistola *et al.*, 1980) and human placental tissues (Turpeenniemi-Hujanen *et al.*, 1981) and for crude lysyl hydroxylase from cells of a patient with the type VI variant of the Ehlers-Danlos syndrome (Turpeenniemi-Hujanen *et al.*, 1981). These data strongly argue against the existence of collagen type-specific lysyl hydroxylase isoenzymes.

Recent studies on the characterization of lysyl hydroxylase by using antiserum against the pure enzyme from chick embryos have indicated that various tissues of the embryo contain an immunologically similar enzyme form when studied by immunodiffusion, and that essentially identical amounts of the antiserum are required for 40% inhibition of the same amount of lysyl hydroxylase activity units from a number of tissues (Turpeenniemi-Hujanen, 1981). These data together with the present results make it highly unlikely that lysyl hydroxylase has collagen-type-specific or tissue-specific isoenzymes with significantly different catalytic or molecular properties. Nevertheless, additional studies with other techniques would be

Table 3. *Hydroxylysine formation in type I and type IV procollagen with 'type I', 'II' and 'IV' lysyl hydroxylases*
The activity of 'type I', 'II' and 'IV' enzymes was tested with type-I procollagen substrate from chick-embryo tendon cells. The same amounts of activity units of the three enzymes were taken and assayed with type-IV procollagen substrate from HT-1080 sarcoma cells.

Enzyme source	Hydroxylysine formed with	
	Type I procollagen (d.p.m./30 min)*	Type IV procollagen (d.p.m./30 min)*
Chick embryo tendon ('type I')	6600	3780
Chick embryo sternum ('type II')	6600	3600
HT-1080 sarcoma cell ('type IV')	6600	3550

* The d.p.m. values can be compared only with the same substrate.

required to demonstrate whether lysyl hydroxylase has any isoenzymes at all.

On the basis of the above conclusions it seems likely that the differences in the extents of lysyl hydroxylation between the genetically distinct collagen types are due to regulation of the amounts of a single type of the enzyme protein in various cells. This would agree with data indicating that differences in the extent of lysyl hydroxylation between type I, II and IV procollagens can be explained to a large extent by differences in the amounts of lysyl hydroxylase activity in cells synthesizing exclusively one of these three collagen types (Risteli *et al.*, 1979; Pihlajaniemi *et al.*, 1981), the assays being carried out with type-I procollagen substrate.

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References

- Alitalo, K., Vaheri, A., Krieg, T. & Timpl, R. (1980) *Eur. J. Biochem.* **109**, 247–255
- Berg, R. A. & Prockop, D. J. (1973) *Biochemistry* **12**, 3395–3401
- Bornstein, P. & Sage, H. (1980) *Annu. Rev. Biochem.* **49**, 957–1003
- Brul, S. D., Bradley, K. H., Hance, A. J., Schafer, M. P., Berg, R. A. & Crystal, R. G. (1980) *J. Biol. Chem.* **255**, 5250–5260
- Dehm, P. & Prockop, D. J. (1972) *Biochim. Biophys. Acta* **264**, 375–382
- Kivirikko, K. I. & Myllylä, R. (1980) in *The Enzymology of Post-Translational Modifications of Proteins* (Freedman, R. B. & Hawkins, H. C., eds.), pp. 53–104, Academic Press, London
- Kivirikko, K. I. & Prockop, D. J. (1972) *Biochim. Biophys. Acta* **258**, 366–379
- Krane, S. M. (1980) in *Gene Families of Collagen and Other Proteins* (Prockop, D. J. & Champe, P. C., eds.), pp. 57–69, Elsevier/North-Holland, New York
- Pihlajaniemi, T., Myllylä, R., Alitalo, K., Vaheri, A. & Kivirikko, K. I. (1981) *Biochemistry* in the press
- Pinnell, S. R., Krane, S. M., Kenzora, J. E. & Glimcher, M. J. (1972) *N. Engl. J. Med.* **286**, 1013–1020
- Plowman, K. M. (1972) *Enzyme Kinetics* pp. 171, McGraw-Hill, New York
- Puistola, U., Turpeenniemi-Hujanen, T. M., Myllylä, R. & Kivirikko, K. I. (1980) *Biochim. Biophys. Acta* **661**, 40–50
- Risteli, J. & Kivirikko, K. I. (1976) *Biochem. J.* **158**, 361–367
- Risteli, L., Risteli, J., Salo, L. & Kivirikko, K. I. (1979) *Eur. J. Biochem.* **97**, 297–303
- Ryhänen, L. & Kivirikko, K. I. (1974) *Biochim. Biophys. Acta* **343**, 129–137
- Turpeenniemi-Hujanen, T. M. (1981) *Biochem. J.* **195**, 669–676
- Turpeenniemi-Hujanen, T. M., Puistola, U. & Kivirikko, K. I. (1980) *Biochem. J.* **189**, 247–253
- Turpeenniemi-Hujanen, T. M., Puistola, U. & Kivirikko, K. I. (1981) *Collagen Relat. Res. Clin. Exp.* **1**, 355–356