Failure of highly purified lysyl hydroxylase to hydroxylate lysyl residues in the non-helical regions of collagen

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The activity of highly purified lysyl hydroxylase towards lysyl residues within both the helical and the *N*-terminal non-helical telopeptide regions of chick type I collagen has been examined. The peptides $\alpha 1(I)$ -CB1 and $\alpha 2(I)$ -CB1, isolated from procollagen following CNBr digestion and containing the *N*-terminal telopeptidyl lysyl residues, failed themselves to act as substrates. With procollagen as substrate, analysis of products obtained following bacterial collagenase digestion of the reaction mixture showed that overall 37% hydroxylation of lysyl residues within the helical region of collagen had been obtained, which may be maximal. No hydroxylation, however, of the single lysyl residue in either $\alpha 1(I)$ -CB1 or $\alpha 2(I)$ -CB1, isolated following CNBr digestion of the reaction mixture, was observed, despite the known susceptibility of these residues to hydroxylation. These findings provide strong circumstantial evidence for the suggestion that a lysyl hydroxylase specific for the telopeptidyl residues and distinct from that active towards lysyl residues in the helical portion of the molecule may exist [Barnes, Constable, Morton & Royce (1974) *Biochem. J.* 139, 461–468].

The biosynthesis of collagen is characterized by several post-translational modifications, one of which is the hydroxylation of certain lysyl residues by lysyl hydroxylase (peptidyl-lysine, 2-oxoglutarate:oxygen 5-oxidoreductase, EC 1.14.11.4), (Prockop *et al.*, 1976, 1979; Kivirikko & Myllylä, 1980, 1982a). Extensive variation is displayed in the extent of lysyl hydroxylation in different collagen types and within the same collagen type from different tissues, or from the same tissue from different species; nevertheless, in any particular situation, the extent of hydroxylation appears to be extremely consistent, implying strict control of this process (Barnes *et al.*, 1974; Kivirikko & Myllylä, 1980, 1982a). Within the helical region of the collagen molecule, hydroxylysine occurs exclusively in the Yaa position of the repeating Xaa-Yaa-Gly triplets that comprise the primary structure of each of the three constituent α chains. However, in the *N*- and *C*-terminal non-helical telopeptide regions of each α chain, where glycine does not constitute every third residue, a single lysyl residue exists that generally occurs, depending upon the

collagen in question, in the sequence Xaa-Lys-Ser or Xaa-Lys-Ala, but which is, nevertheless, susceptible to hydroxylation (Barnes *et al.*, 1974; Kivirikko & Myllylä, 1980, 1982a; Galloway, 1982). Control of hydroxylation at this particular site is especially important, since the residue in question participates in the formation of intermolecular crosslinks following its oxidative deamination to the corresponding aldehyde by lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13), (Siegel, 1979), and the extent of hydroxylation at this site determines the nature of the crosslinks formed (Bailey *et al.*, 1974; Barnes *et al.*, 1974; Robins, 1982). Crosslink formation involving the interaction of a hydroxylysyl aldehyde residue with either a lysyl or hydroxylysyl residue within the triple-helical region of an adjacent molecule results in the formation of an aldimine bond that can undergo an Amadori rearrangement to a keto-imine form (Bailey *et al.*, 1974; Tanzer, 1976; Robins, 1982), which may be the precursor of the mature 3-hydroxypyridinium crosslinks (Robins, 1982; Eyre *et al.*, 1984).

A detailed study of the extent of lysyl hydroxylation in the *N*-terminal telopeptides of type I collagen from different tissues of the chick in relation to age indicated that variations at these

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sites were unrelated to variations in the corresponding whole chain values for the extent of lysyl hydroxylation (Barnes *et al.*, 1974). Thus, for example, whilst there is a complete disappearance of hydroxylysine in the telopeptides of type I collagen from skin with age, there is a decline in the overall extent of hydroxylation which is somewhat less than that occurring in type I collagen from bone, where, paradoxically, the extent of telopeptidyl lysyl hydroxylation remains relatively high. In view of this, and of the atypical primary structure of the telopeptides, it was consequently suggested that hydroxylation of telopeptidyl lysyl residues might be effected by a specific lysyl hydroxylase distinct from that active towards lysyl residues within the helical regions of the α chains.

In the present study, the activity of highly purified lysyl hydroxylase towards lysyl residues within both the helical and the *N*-terminal non-helical telopeptide regions of type I procollagen, the unhydroxylated form of procollagen, has been examined, and the results provide strong circumstantial evidence for the existence of a telopeptidyl-specific lysyl hydroxylase.

A brief account of this work has already been given (Royce & Barnes, 1984).

Experimental

Materials

Fertilized eggs were obtained from Winter Egg Farm, Thriplow, Cambs., U.K., and incubated in a humidified atmosphere at 37°C in a Western incubator until embryos reached the desired age. Concanavalin A-Sepharose 4B and CNBr-activated Sepharose 4B were from Pharmacia, Uppsala, Sweden. Citrate-soluble rat skin collagen (Gallop & Seifter, 1963; Bazin & Delaunay, 1976), denatured by heating at 60°C for 15 min and then cooled, was coupled to CNBr-activated Sepharose 4B according to the manufacturer's recommended procedure (*Affinity Chromatography: Principles and Methods*). The collagen content of the product, termed 'collagen-Sepharose', was 4.8 mg/ml of packed gel, as determined by hydrolysing a sample of the gel and measuring the hydroxyproline released by the method of Stegemann & Stalder (1967). L-[4,5-³H]Lysine monohydrochloride (sp. radioactivity 75–100 Ci/mmol) and L-[G-³H]tryptophan (sp. radioactivity 4.7 Ci/mmol) were purchased from Amersham International. Bacterial collagenase from *Clostridium histolyticum* (CLSPA; Millipore) was further purified by chromatography on DEAE-cellulose (Whatman DE-52) and Sephadex G-200 (Pharmacia) as described by Lee-Own & Anderson (1975). That this highly purified preparation was free of non-

specific proteinase activity was demonstrated by the fact that it caused the release of <1% of the radioactivity from a [³H]tryptophan-labelled protein substrate, prepared as described by Lee-Own & Anderson (1975), into trichloroacetic acid-soluble peptides over a period of 22 h at 37°C. Silicone-treated glassware was used at all stages in the preparation and use of procollagen.

Methods

All procedures in the description of methods below were performed at 4°C, except where otherwise stated.

Purification of lysyl hydroxylase

Highly purified lysyl hydroxylase was obtained from 14-day old whole chick embryos, basically as described by Turpeenniemi-Hujanen *et al.* (1980, 1981) and Kivirikko & Myllylä (1982b). Briefly, embryos were homogenized in 0.2 M-NaCl/0.1 M-glycine/10 μ M-dithiothreitol/0.1% Triton X-100/20 mM-Tris/HCl, pH 7.5 at 4°C (Ryhänen, 1976). That fraction of the 15000g supernatant precipitating between 17 and 55% (preparation I) or 0 and 55% (preparation II) (NH₄)₂SO₄ saturation, was dissolved in 'enzyme buffer', (0.2 M-NaCl/0.1 M-glycine/10 μ M-dithiothreitol/1% glycerol/20 mM-Tris/HCl, pH 7.5 at 4°C), containing 3 mM-MnCl₂ and dialysed against the same buffer. After removal of insoluble material by centrifugation, concanavalin A-Sepharose, previously equilibrated with enzyme buffer containing 3 mM-MnCl₂, was added to the sample and the mixture was stirred gently for 18 h. The resin was subsequently washed with enzyme buffer containing 3 mM-MnCl₂ and 0.5 M-methyl α -D-glucoside, then poured into a column. Washing was continued until the A₂₂₅ of the eluate was 0.1–0.3 and the enzyme was subsequently eluted with enzyme buffer containing 0.3 M-methyl α -D-glucoside and 60% (v/v) ethylene glycol. Enzyme buffer was added until the ethylene glycol concentration was 20% (v/v), following which collagen-Sepharose, previously equilibrated with enzyme buffer and heated to 60°C for 20 min then cooled immediately before use, was added and the mixture was stirred gently for 18 h. The resin was then poured into a column, washed with enzyme buffer and eluted with enzyme buffer containing 60% (v/v) ethylene glycol. The enzyme preparations, termed 'highly purified' lysyl hydroxylase (Kivirikko & Myllylä, 1982b), were concentrated in an Amicon ultrafiltration cell with a PM-30 membrane and stored at –20°C. Following denaturation and reduction with mercaptoethanol, analysis of the two enzyme preparations by electrophoresis in sodium dodecyl sulphate/6% polyacrylamide-slab gels, using the method of Laemmli (1970) and staining with

Coomassie Brilliant Blue, indicated that preparation I was homogeneous, whilst preparation II showed two minor contaminants. Lysyl hydroxylase activity was assayed as described below, using L-[4,5-³H]lysine-labelled procollagen substrate.

Preparation of substrates

Procollagen. L-[4,5-³H]Lysine-labelled procollagen was prepared from the frontal bones of 16-day old chick embryos. The bones from batches of 24 embryos were preincubated for 30 min at 37°C in 14 ml of modified Krebs' medium II (Krebs, 1950; Kivirikko & Myllylä, 1982b) containing 0.3 mM- α,α' -dipyridyl and β -aminopropionitrile fumarate (50 μ g/ml). L-[4,5-³H]Lysine monohydrochloride was added to a final concentration of 50 μ Ci/ml and the incubation was continued for a further 3 h. The bones were washed in cold water, blotted, frozen in liquid N₂, then crushed in a stainless steel mortar. The crushed bones were extracted for 16 h with 1 M-NaCl/50 mM-Tris/HCl, pH 7.5 at 4°C, containing EDTA (10 mM), *N*-ethylmaleimide (10 mM) and phenylmethanesulphonyl fluoride (0.86 mM). The extract was centrifuged (30 000 *g* for 50 min) and solid NaCl was slowly added to the supernatant to a final concentration of 20% (w/v). The precipitate, collected by centrifugation (30 000 *g* for 50 min), was resuspended in 0.2 M-NaCl/50 mM-Tris/HCl, pH 7.5 at 4°C, and dialysed against the same solution, pH 7.8 at 4°C. The retentate was left at 37°C for 105 min to allow unlabelled soluble collagen to form fibrils (Peterkofsky & DiBlasio, 1975) which were removed by centrifugation (30 000 *g* for 45 min at 22°C), and the supernatant was used as the procollagen substrate.

***N*-Terminal telopeptides.** Peptides α 1(I)-CB1 and α 2(I)-CB1, consisting mainly of the *N*-terminal telopeptides, were isolated from the procollagen substrate following CNBr digestion using the conditions of Scott & Veis (1976). Procollagen was dialysed into formic acid (70%, v/v), CNBr was added and incubation was performed for 6 h at 30°C. Reactions were terminated by the addition of a 10-fold excess of water and excess CNBr was removed by lyophilization. Digests were lyophilized from 0.15 M-acetic acid to ensure complete removal of volatile products and were then taken up in 0.15 M-acetic acid and dialysed exhaustively against water. The diffusate was lyophilized, taken up in 0.1 M-acetic acid and applied to a column (2.5 cm $\times$ 60 cm) of Sephadex G-15 (Pharmacia) and eluted with the same solution; that portion of the eluate containing radioactivity was then lyophilized. α 1(I)-CB1 and α 2(I)-CB1 were then isolated from the diffusates of the CNBr digests by chromatography on phosphocellulose (Whatman) as described previously (Barnes *et al.*, 1974),

lyophilized, desalted on Sephadex G-15, (as above), and lyophilized. Prior to use as substrates they were taken up in 0.2 M-NaCl/50 mM-Tris/HCl, pH 7.8 at 4°C.

Assay of lysyl hydroxylase

Lysyl hydroxylase activity was assayed using L-[4,5-³H]lysine-labelled procollagen substrate, by the ³H-release procedure of Miller (1972). Cofactors and reaction mixtures were as described by Kivirikko & Myllylä (1982b). The amount of sample assayed was such that the final concentration of ethylene glycol in the reaction mixture was not greater than 6% (v/v) because of its inhibitory effect. The ³H₂O formed during the reaction was determined as described by Royce *et al.* (1980) with appropriate volume adjustments. Correction was made for non-specific background release of radioactivity.

Determination of hydroxylysine formation in substrates

Highly purified lysyl hydroxylase was incubated with the isolated peptides α 1(I)-CB1 and α 2(I)-CB1 as described above, except that the incubation period was 5 h. Reaction mixtures were subsequently hydrolysed in 6 M-HCl at 110°C for 24 h. To investigate the activity of highly purified lysyl hydroxylase towards the *N*-terminal telopeptidyl lysyl residues in intact procollagen, procollagen itself was used as substrate. Reaction mixtures of 10 ml volume were used and the incubation period was increased to 7 h. Peptides α 1(I)-CB1 and α 2(I)-CB1 were subsequently isolated following CNBr digestion of the reaction mixture as described above, and hydrolysed. Hydrolysates were dried and washed once with water. Hydroxyl[³H]lysine in the hydrolysates was separated from [³H]lysine by ion-exchange chromatography on a column (1 cm $\times$ 22 cm) of Dowex resin (AG-50W-X8; 200–400 mesh, H⁺ form; Bio-Rad) at 50°C, eluting with 0.3 M-sodium citrate buffer, pH 4.8 (Piez & Likins, 1957). Unlabelled hydroxylysine was added to each hydrolysate prior to chromatography and its elution position was determined colorimetrically by the semi-micro procedure of Blumenkrantz & Asboe-Hansen (1973). With each incubation, control samples were included in which the highly purified lysyl hydroxylase was incubated with procollagen and ³H₂O formation was determined as a check of enzyme activity. The activity of the lysyl hydroxylase preparations towards the helical portions of the α chains of procollagen was determined in these control reaction mixtures. The pellets obtained following addition of trichloroacetic acid to the reaction mixtures were washed three times with ice-cold trichloroacetic acid (5%, w/v), dissolved in 0.2 M-

NaOH and subjected to digestion with highly purified bacterial collagenase as described by Peterkofsky (1972). Collagenase-released material was hydrolysed and chromatographed as described above. The extent of hydroxylation of substrates was calculated from the distribution of radioactivity between hydroxy[^3H]lysine and [^3H]lysine, correction being made for the 25% loss of radioactivity that occurs when [4,5- ^3H]lysine in collagen is converted to hydroxylysine (Popenoe *et al.*, 1965).

Results and discussion

Isolated peptides $\alpha 1(\text{I})$ -CB1 and $\alpha 2(\text{I})$ -CB1 as substrates

With neither of the two peptides $\alpha 1(\text{I})$ -CB1 or $\alpha 2(\text{I})$ -CB1, each containing a single lysyl residue in the sequences Xaa-Lys-Ser and Xaa-Lys-Ala, respectively, was any formation of hydroxylysine detected following incubation with highly purified lysyl hydroxylase. This might indicate a requirement for a telopeptidyl-specific lysyl hydroxylase, but it could also be argued that a portion of the repeating Gly-Xaa-Yaa sequence of the helical region of the α chains is required for lysyl hydroxylase to bind to. The telopeptides themselves have an atypical amino acid sequence, lacking the repeating Gly-Xaa-Yaa triplets characteristic of the helical portion of the α chains (Bornstein & Traub, 1979; Galloway, 1982). Although the minimum sequence requirement for lysyl hydroxylase activity is an Xaa-Lys-Gly triplet, increasing chain length of synthetic peptide substrates of repeating Xaa-Yaa-Gly sequences up to about 30 residues containing one or more Yaa position lysyl residues is associated with a decrease in K_m values (Kivirikko *et al.*, 1972; Kivirikko & Myllylä, 1980; Kikuchi & Tamiya, 1982). Since peptide $\alpha 1(\text{I})$ -CB1 consists of 17 residues and $\alpha 2(\text{I})$ -CB1 of 15 (Kang & Gross, 1970; Galloway, 1982) it is unlikely that their failure to act as substrates is merely a feature of chain length. In this context, it is relevant that a partially purified preparation of lysyl hydroxylase showed activity towards the nonapeptide [Lys 8]vasopressin lacking Gly-Xaa-Yaa triplets (Kivirikko *et al.*, 1972), but this might be explained by the fact that the single lysyl residue exists in the Yaa position of an Xaa-Yaa-Gly sequence. Interestingly, partially purified lysyl hydroxylase does hydroxylate not only the 212-residue lysine-rich histone containing lysyl residues in Xaa-Lys-Gly sequences, but also the 135-residue arginine-rich histone which contains lysyl residues entirely in sequences other than Xaa-Lys-Gly, some of which are in the Xaa-Lys-Ser and Xaa-Lys-Ala sequences apparent in the telopeptides (Ryhänen, 1975). However, since the enzyme

used in those studies was only partially purified, it is not possible to say whether more than one lysyl hydroxylase was present.

Protocollagen as substrate

When highly purified preparations of lysyl hydroxylase were incubated with protocollagen, analysis of peptides released by digestion of the reaction mixtures with highly purified bacterial collagenase showed that the formation of hydroxylysine had occurred in the helical portions of the α chains (Table 1). Consideration of lysyl or hydroxylysyl residues within the helical portion of the *N*-terminal propeptide, peptide Col 3, which is also susceptible to bacterial collagenase digestion, would have negligible impact upon these results (Fessler & Fessler, 1978; Galloway, 1982). Enzyme preparation II, which showed some inhomogeneity upon electrophoretic analysis, was less active than preparation I. With two different protocollagen preparations, highly purified lysyl hydroxylase preparation I hydroxylated lysyl residues within the helical regions of the α chains to the extent of 36–37%, which is equal to the highest levels of hydroxylation observed in type I collagen from embryonic chick frontal bones *in vitro* (Barnes *et al.*, 1974). Since only lysyl residues in the Yaa position of certain of the repeating Xaa-Yaa-Gly triplets are susceptible to hydroxylation (Kivirikko & Myllylä, 1980, 1982a), this extent may be maximal. The complete amino acid sequence of the chick $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$ chains has not yet been fully elucidated (Galloway, 1982). The maximum elevation of the extent of hydroxylation of acid-soluble type I collagen from calf skin obtained upon incubation with partially purified

Table 1. *Extent of hydroxylation of lysyl residues within the helical and the N-terminal non-helical telopeptide regions of protocollagen following incubation with highly purified lysyl hydroxylase*

Following incubation of [4,5- ^3H]lysine-labelled protocollagen with highly purified lysyl hydroxylase, the extent of hydroxylation of lysyl residues was determined from the distribution of radioactivity between lysine and hydroxylysine; in the helical regions, containing the majority of the lysyl residues, by analysis of products released by digestion of the reaction mixture with highly purified bacterial collagenase, and in the telopeptides following their isolation after digestion of the reaction mixture with CNBr, as the peptides $\alpha 1(\text{I})$ -CB1 and $\alpha 2(\text{I})$ -CB1.

Enzyme preparation	Extent of hydroxylation (%)		
	Helical region	$\alpha 1(\text{I})$ -CB1	$\alpha 2(\text{I})$ -CB1
I	36.6	0	0
II	9.8	0	0

lysyl hydroxylase was from approx. 20% to 44% (Kivirikko *et al.*, 1973), of the same order as observed here.

In contrast, however, no hydroxylysine formation was detected in the *N*-terminal non-helical telopeptide regions in $\alpha 1(\text{I})$ -CB1 and $\alpha 2(\text{I})$ -CB1, isolated from the procollagen following CNBr digestion of the reaction mixtures (Table 1), despite the fact that relatively high levels of hydroxylation have been reported at these sites in chick bone collagen *in vivo* (Lane & Miller, 1969), and in collagen synthesized by embryonic chick frontal bones *in vitro* (Barnes *et al.*, 1974). This finding is of particular interest in relation to the earlier observation that a crude extract of embryonic chick tibiae was capable of hydroxylating the *N*-terminal telopeptidyl lysyl residues in chick embryo tendon collagen (Barnes *et al.*, 1971) and provides strong circumstantial evidence for the existence of a telopeptidyl-specific lysyl hydroxylase.

Lysyl hydroxylase is located within the cisternae of the rough endoplasmic reticulum, hydroxylation of lysyl residues occurring both in nascent pro- α chains and in complete pro- α chains after their release from ribosomes (Kivirikko & Myllylä, 1980, 1982a). It has been shown that when tissues or cells are incubated in the presence of a chelator of Fe^{2+} , or under anaerobic conditions, Fe^{2+} and O_2 being cofactors of both lysyl and prolyl hydroxylases, the completed non-hydroxylated pro- α chains which are released from the ribosomes and which accumulate within the cisternae of the rough endoplasmic reticulum can be hydroxylated upon reversal of the inhibition (Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980, 1982a). Thus, the procollagen used in the present study would be expected to have been a suitable substrate for the hydroxylation of telopeptidyl residues, and, indeed, the hydroxylation of lysyl residues which occurred within the helical regions of the α chains would support this expectation.

In the chick, whereas peptide $\alpha 1(\text{I})$ -CB1 is separated from the *N*-terminal procollagen peptide by the dipeptide $\alpha 1(\text{I})$ -CB0, in the case of $\alpha 2(\text{I})$ -CB1 there is no methionyl residue separating it from the *N*-terminal procollagen peptide (Bornstein & Traub, 1979; Galloway, 1982) and thus it would not be expected to result from CNBr cleavage of procollagen. In the studies described here, $\alpha 1(\text{I})$ -CB1 and $\alpha 2(\text{I})$ -CB1 were obtained from procollagen in a ratio of 5.5:1, which would indicate that the *N*-terminal propeptides had been cleaved from approx. 30% of the procollagen.

Additional circumstantial evidence for the existence of a telopeptidyl-specific lysyl hydroxylase is provided by the observation that whilst the overall extent of lysyl hydroxylation in collagen synthe-

sized by mouse 3T6 cells is largely unimpaired in ascorbate-deficient cultures, there is an alteration in the crosslink pattern from (after reduction) dihydroxylysinonorleucine to hydroxylysinonorleucine (Bates *et al.*, 1972), which strongly indicates a substantial reduction of hydroxylation in the telopeptides.

The telopeptidyl lysyl residue within peptide $\alpha 2(\text{I})$ -CB1 of some species other than the chick does exist within an Xaa-Lys-Gly sequence (Bornstein & Traub, 1979; Galloway, 1982). It seems reasonable to suppose that a telopeptidyl-specific lysyl hydroxylase would, in view of the atypical primary structure of the telopeptides, with their lack of repeating Gly-Xaa-Yaa triplets, be active towards telopeptidyl residues within Xaa-Lys-Gly sequences as well as those within the Xaa-Lys-Ser and Xaa-Lys-Ala sequences examined here.

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