Further Studies on the Effect of the Collagen Triple-Helix Formation on the Hydroxylation of Lysine and the Glycosylations of Hydroxylysine in Chick-Embryo Tendon and Cartilage Cells

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The hydroxylation of lysine and glycosylations of hydroxylysine were studied in isolated chick-embryo tendon and cartilage cells under conditions in which collagen triple-helix formation was either inhibited or accelerated. The former situation was obtained by incubating the tendon cells with 0.6 mM-dithiothreitol, thus decreasing their proline hydroxylase activity by about 99%. After labelling with [^{14}C]proline, the formation of hydroxy[^{14}C]proline was found to have declined by about 95%. Since the hydroxylation of a relatively large number of proline residues is required for triple-helix formation at 37°C, the pro- α -chains synthesized under these conditions apparently cannot form triple-helical molecules. Labelling experiments with [^{14}C]lysine indicated that the degree of hydroxylation of the lysine residues in the collagen synthesized was slightly increased and the degree of the glycosylations of the hydroxylysine residues more than doubled, the largest increase being in the content of glucosylgalactosylhydroxylysine. Recovery of chick-embryo cartilage cells from temporary anoxia was used to obtain accelerated triple-helix formation. A marked decrease was found in the extent of hydroxylation of the lysine residues in the collagen synthesized under these conditions, and an even larger decrease occurred in the glycosylations of the hydroxylysine residues. The results support the previous suggestion that the triple-helix formation of the pro- α -chains prevents further hydroxylation of lysine residues and glycosylations of hydroxylysine residues during collagen biosynthesis.

The hydroxylysine, galactosylhydroxylysine and glucosylgalactosylhydroxylysine of collagen are synthesized as post-translational modifications of the procollagen polypeptide chains catalysed by the enzymes lysine hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase (for reviews on collagen biosynthesis, see Martin *et al.*, 1975; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976). These reactions are initiated while the polypeptide chains are growing on the ribosomes, and continue after the release of completed chains into the cisternae of the endoplasmic reticulum (Uitto & Prockop, 1974a; Harwood *et al.*, 1975a,b; Brownell & Veis, 1975; Oikarinen *et al.*, 1976a,b).

Experiments *in vitro* with lysine hydroxylase (Kivirikko *et al.*, 1973; Ryhänen & Kivirikko, 1974), collagen galactosyltransferase (Risteli *et al.*, 1976) and collagen glucosyltransferase (Myllylä *et al.*, 1975b) indicate that although these enzymes readily act on denatured substrate polypeptide chains, they do not catalyse the reactions with native triple-helical molecules. Studies with isolated cells on the time course of the hydroxylation of lysine residues and glycosylations of hydroxylysine residues (Oikarinen

et al., 1976a), and on the effect of L-azetidine-2-carboxylic acid on these reactions (Oikarinen *et al.*, 1976b), also suggest that these reactions do not continue after the triple-helix formation even in intact cells.

The formation of the collagen triple-helix seems to have two requirements. An appropriate number of proline residues must be hydroxylated (Berg & Prockop, 1973a,b; Jimenez *et al.*, 1973; Sakakibara *et al.*, 1973; Ward & Mason, 1973; Fessler & Fessler, 1974; Uitto & Prockop, 1974b), and the interchain disulphide bonds must presumably be formed (Uitto & Prockop, 1973, 1974c) before this process can take place. Active proline hydroxylase tetramers can be dissociated into inactive monomers by treatment with dithiothreitol both *in vitro* (Berg & Prockop, 1973c; Tuderman *et al.*, 1975b) and in intact cells (Stassen *et al.*, 1973), and also interchain disulphide bonding can be prevented in intact cells by treatment with this compound (Uitto & Prockop, 1973). Accordingly, the formation of the collagen triple helix can be effectively inhibited by such treatment. In the present work, this approach was used to study further the effect of the inhibition of collagen triple-helix

formation on the extent of the hydroxylation of lysine residues and the glycosylations of hydroxy-lysine residues in isolated chick-embryo tendon cells.

The hydroxylation of proline residues occurs earlier than the formation of the interchain disulphide bonds during collagen biosynthesis in isolated chick-embryo cartilage cells (Uitto & Prockop, 1974c; Harwood *et al.*, 1975a), so that the latter process seems to be rate-limiting for the triple-helix formation. It has previously been demonstrated that if the cells are allowed to accumulate non-hydroxylated pro- α -chains under conditions in which the hydroxylation reactions are inhibited, the formation of interchain disulphide bonds takes place during this time period, and on subsequent reversal of the inhibition, hydroxylation of proline residues and triple-helix formation both occur at a rapid rate (Jimenez *et al.*, 1973; Uitto & Prockop, 1974d). Under such conditions, more limited time is available for the hydroxylation of lysine residues, and the collagen synthesized contains a smaller amount of hydroxylysine (Uitto & Prockop, 1974d). This effect is even more marked when the temperature of the cells is lowered during reversal of the inhibition (Uitto & Prockop, 1974d). In the present work, this experimental design was used to study the effect of accelerated triple-helix formation on the extent of the glycosylations of the hydroxy-lysine residues in the collagen synthesized by isolated chick-embryo cartilage cells.

Experimental

Materials

Fertilized eggs of white Leghorn chickens were obtained from Siipikarjanhoitajien liitto r.y. (Hämeenlinna, Finland) and incubated in a moist atmosphere at 37°C until used.

[14 C]Proline and [14 C]lysine were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.), purified bacterial collagenase was from Sigma Chemical Co. (Kingston-upon-Thames, Surrey KT2 7BH, U.K.), and trypsin [2.5% (w/v) solution in 0.9% NaCl] was from Gibco Corp. (Grand Island, NY, U.S.A.). Foetal calf serum was purchased from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.).

Isolation and incubation of matrix-free cells from tendon and cartilage

The cells were isolated from the leg tendons and sterna of 17-day chick embryos by enzymic digestion with trypsin and collagenase (Dehm & Prockop, 1971, 1973), and tendon cells were incubated in a modified Krebs medium without the addition of foetal calf serum, at 37°C (Dehm & Prockop, 1971). Cartilage cells were incubated in a modified Krebs medium containing 20% (w/v) foetal calf serum with moderate agitation at 33°C (Dehm & Prockop, 1973).

Radioactively labelled compounds were added as indicated in various experiments and the cells were incubated under the conditions described in the legends to the Figures or Tables. The cells were then separated from the medium by centrifugation at 1200g for 10 min at room temperature, or else the entire incubation system was taken for further processing.

Assays of enzyme activities

Proline hydroxylase and lysine hydroxylase activities were assayed with 60000 d.p.m. of [14 C]-proline- or 110000 d.p.m. of [14 C]lysine-labelled protocollagen substrate (Kivirikko & Prockop, 1972; Tuderman *et al.*, 1975a). Collagen glycosyltransferase activities were assayed as described previously (Myllylä *et al.*, 1975a), except that 30 μ M-UDP-[14 C]galactose [New England Nuclear; diluted with unlabelled compound (Sigma) to a specific radioactivity of 15 Ci/mol] was used for the assay of galactosyltransferase activity and 60 μ M-UDP-[14 C]glucose [New England Nuclear; diluted with unlabelled compound (Sigma) to a specific radioactivity of 6.6 Ci/mol] for that of glucosyltransferase activity.

Other assays

After incubation with [14 C]proline the samples were dialysed against running tap water for 24–48 h and the materials retained were hydrolysed in 6M-HCl in sealed tubes overnight at 120°C. The hydrolysed samples were evaporated to dryness on a steam bath and dissolved in 4 ml of water. The hydroxy-[14 C]proline content was assayed by a specific radiochemical procedure (Juva & Prockop, 1966).

After incubation with [14 C]lysine, the samples were dialysed against running tap water as above. In some experiments total 14 C radioactivity and hydroxy[14 C]-lysine were assayed by a specific method (Blumenkrantz & Prockop, 1969), and in others total radioactivity, hydroxy[14 C]lysine and glycosylated hydroxy[14 C]lysine were assayed after alkaline hydrolysis and brief ion-exchange chromatography (Myllylä *et al.*, 1975a) by amino acid analyser as described previously (Askenasi, 1973; Oikarinen *et al.*, 1976a). The digestion of radioactive protein with highly purified bacterial collagenase (Sigma) was carried out for 24 h at 37°C in a solution consisting of 0.02M-NH₄HCO₃ and 0.1 mM-CaCl₂, pH 7.5 (see Myllylä *et al.*, 1975b). The diffusible peptides were then hydrolysed with 6M-HCl and assayed for total radioactivity and total hydroxy[14 C]lysine.

Sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis was carried out by using 6% (w/v) acrylamide as described by King & Laemmli (1971). The radioactivity was then determined by fluoro-

graphy (Bonner & Laskey, 1974). Before the gel electrophoresis the samples were freeze-dried, dissolved in 0.5 ml of the 'sample buffer' (King & Laemmli, 1971) and heated for 3 min at 100°C.

All ^{14}C radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 80% and a background of 25 c.p.m., in the scintillants described previously (Myllylä *et al.*, 1975a; Oikarinen *et al.*, 1976a).

Results

Effect of dithiothreitol on the activities of proline hydroxylase, lysine hydroxylase and collagen glycosyltransferases in tendon cells

The tendon cells were incubated for 30 min in a modified Krebs medium containing various amounts of dithiothreitol, and the enzyme activities determined in the 15000g supernatants of the cell homogenates (Fig. 1). There was a marked decrease in

Fig. 1. *Effect of dithiothreitol on the activities of proline hydroxylase, lysine hydroxylase and collagen glycosyltransferases in isolated tendon cells*

Tendon cells (12×10^7) were incubated for 30 min in 3 ml of modified Krebs medium containing various concentrations of dithiothreitol, after which they were centrifuged at 1200g for 10 min at 4°C, and washed once with the homogenization solution without Triton X-100 and re-centrifuged. The cells were homogenized with 60 strokes of a Teflon/glass homogenizer in 2 ml of a solution containing 0.1 M-glycine, 0.1 M-NaCl, 50 μM -dithiothreitol, 20 mM-Tris/HCl buffer (pH 7.5 at 4°C) and 0.1% Triton X-100. The homogenates were incubated at 4°C for 60 min, and then centrifuged at 15000g for 30 min at 4°C. The protein content was assayed by the method of Lowry *et al.* (1951), and proline hydroxylase ($\bullet$), lysine hydroxylase ($\bullet$), collagen galactosyltransferase ($\square$) and collagen glucosyltransferase ($\blacksquare$) activities were measured as described in the Experimental section. The results are shown as the mean values for duplicate assays and are expressed as percentages of values obtained without the addition of dithiothreitol to the incubation medium.

proline hydroxylase activity to about 1% of the control value with 0.6 mM-dithiothreitol, and lysine hydroxylase activity also diminished considerably, but was still about 60% with 0.6 mM-dithiothreitol. Only slight decreases were found in the two collagen glycosyltransferase activities.

Effect of dithiothreitol on the incorporation of [^{14}C]proline and [^{14}C]lysine and on the formation of hydroxy[^{14}C]proline and hydroxy[^{14}C]lysine in tendon cells

The tendon cells were incubated with [^{14}C]proline or [^{14}C]lysine in a modified Krebs medium containing various amounts of dithiothreitol, and total radioactivity, hydroxy[^{14}C]proline and hydroxy[^{14}C]lysine were assayed (Fig. 2). The incorporation of [^{14}C]proline and [^{14}C]lysine decreased by about 40–55% in experiments with a dithiothreitol concentration of 0.3 or 0.6 mM. The formation of hydroxy[^{14}C]proline decreased even more, and was about 5% of the control value with 0.6 mM-dithiothreitol. By

Fig. 2. *Effect of dithiothreitol on the incorporation of [^{14}C]proline and [^{14}C]lysine and the synthesis of hydroxy[^{14}C]proline and hydroxy[^{14}C]lysine in isolated tendon cells*

Tendon cells (10^7 per sample) were preincubated for 30 min in 2 ml of modified Krebs medium containing various amounts of dithiothreitol. The cells were labelled with 2 μCi of [^{14}C]proline or with 2 μCi of [^{14}C]lysine for 2 h, and protein synthesis was stopped by adding cycloheximide and α -bipyridine. Total radioactivity, hydroxy[^{14}C]proline and hydroxy[^{14}C]lysine were determined as described in the Experimental section. Values are the means for duplicate samples in the same experiment and are expressed as percentages of the values obtained without the addition of dithiothreitol. Total radioactivity in the cells plus medium when labelled with [^{14}C]proline ($\circ$); hydroxy[^{14}C]proline in the cells plus medium ($\bullet$); total radioactivity in the cells plus medium when labelled with [^{14}C]lysine ($\square$); hydroxy[^{14}C]lysine in the cells plus medium ($\blacksquare$).

contrast, the formation of hydroxy[^{14}C]lysine decreased to only about the same extent as the incorporation of [^{14}C]lysine.

The size of the [^{14}C]proline-labelled polypeptide chains synthesized in the presence of 0.6 mM-dithiothreitol for 2 h was studied by using polyacrylamide-slab-gel electrophoresis in the presence of sodium dodecyl sulphate and β -mercaptoethanol, as described in the Experimental section. The percentage of total radioactivity in complete pro- α -chains was found to be similar in the control sample and in that incubated with dithiothreitol.

Hydroxy[^{14}C]lysine and glycosylated hydroxy[^{14}C]lysine contents of collagen synthesized by tendon cells in the presence of dithiothreitol

The tendon cells were preincubated for 30 min in a modified Krebs medium containing 0.6 mM-dithiothreitol, and then incubated with [^{14}C]lysine for an additional 2 h in the presence of this compound. The hydroxy[^{14}C]lysine and glycosylated hydroxy[^{14}C]lysine formed were assayed as described in the Experimental section. Since the ratio of radioactivity in hydroxylysine to total radioactivity may be affected either by the degree of hydroxylation of the lysine residues in the collagen or by the relative amount of collagen synthesis compared with total protein synthesis, the degree of hydroxylation of the lysine residues in the collagen itself was determined by collagenase digestion as described previously (Oikarinen *et al.*, 1976b). The results indicated that the ratio of hydroxy[^{14}C]lysine to total radioactivity in diffusible peptides obtained after collagenase digestion was 0.46 in the protein synthesized by the

control cells and 0.51 in that synthesized in the presence of dithiothreitol. On the basis of this value and the relative amounts of galactosylhydroxy[^{14}C]lysine and glucosylgalactosylhydroxy[^{14}C]lysine within the total hydroxy[^{14}C]lysine assayed without collagenase digestion, it was possible to calculate the values shown in Table 1. There was a slight increase in the total hydroxylysine content, and a marked increase, to about 230%, in the substituted hydroxylysine content of the collagen synthesized in the presence of dithiothreitol. The increase in the disaccharide content was larger than in the monosaccharide content, and about 80% of total carbohydrate units in the collagen synthesized under these conditions were present in the form of disaccharide units.

Effect of temporary anoxia and its reversal on the synthesis of hydroxy[^{14}C]lysine and glycosylated hydroxy[^{14}C]lysine by isolated chick-embryo cartilage cells

The cartilage cells were pulsed with [^{14}C]lysine for 60 min in a nitrogen atmosphere at 33°C and the pulse was chased by adding cycloheximide and unlabelled lysine for a further 20 min under nitrogen. The incubation was then continued for 30 min in air to allow the hydroxylation of the proline and lysine residues, which was inhibited during incubation under nitrogen (see the introduction). Some of the samples were digested with collagenase and used to determine the ratio of hydroxy[^{14}C]lysine to [^{14}C]lysine in collagen (see the preceding paragraph), and the remainder were used to assay the relative amounts of galactosylhydroxy[^{14}C]lysine and glucosylgalactosylhydroxy[^{14}C]lysine.

Table 1. *Hydroxylysine and glycosylated hydroxylysine content of the collagen synthesized by isolated chick-embryo tendon cells in the presence or absence of 0.6 mM-dithiothreitol*

The tendon cells (10^6) were preincubated in a modified Krebs medium with or without 0.6 mM-dithiothreitol for 30 min at 37°C. [^{14}C]Lysine (20 μCi) was added and the cells were incubated for 2 h, collagen and protein synthesis being stopped by adding cycloheximide and $\alpha\alpha$ -bipyridine. After dialysis under running tap water, some of the sample was digested with collagenase, and hydroxy[^{14}C]lysine and [^{14}C]lysine were determined in the diffusible peptides by amino acid analyser after HCl hydrolysis, as described in the text. The remainder of the sample was hydrolysed with NaOH, and hydroxy[^{14}C]lysine, substituted hydroxy[^{14}C]lysine and [^{14}C]lysine were determined by amino acid analyser. The calculations were performed as described by Oikarinen *et al.* (1976b), assuming the sum of lysine plus hydroxylysine to be 35 residues per 1000 (Berg & Prockop, 1973b). The values for citrate-soluble rat skin collagen were calculated from the data of Spiro (1969), as described by Oikarinen *et al.* (1976b).

Amino acid	Content (residues/1000 residues)		
	Citrate-soluble rat skin collagen	Collagen synthesized by the tendon cells	
		Control	Dithiothreitol
Total hydroxylysine	7.6	16	18
Substituted hydroxylysine	1.3	4.6	10.8
Monosaccharide	0.5	1.4	2.3
Disaccharide	0.8	3.2	8.5
Lysine	27.4	19	17
Hydroxylysine plus lysine	35	35	35

Table 2. *Hydroxylysine and glycosylated hydroxylysine content of the collagen synthesized by isolated chick-embryo cartilage cells subjected to temporary anoxia*

Cartilage cells from the sterna of 20 17-day chick embryos were divided between two Erlenmeyer flasks. One of these flasks was preincubated for 30 min under N₂, containing 15 ml of modified Krebs medium containing 20% (w/v) foetal calf serum at 33°C. Then 0.5 ml of modified Krebs medium containing 20 μ Ci of [¹⁴C]lysine was added and the incubation was continued for 60 min. The pulse was stopped by adding 1.5 mg of cycloheximide and 5 mg of unlabelled lysine in 0.2 ml of modified Krebs medium, and the incubation was continued for a further 20 min under N₂. Finally the incubation was continued for a further 30 min in air and the cells were then cooled in an ice bath. α -Bipyridine was added in modified Krebs medium to provide a concentration of 1 μ mol/ml. The control cells were also labelled for 60 min with 20 μ Ci of [¹⁴C]lysine at 33°C, and 1.5 mg of cycloheximide and 5 mg of unlabelled lysine were added and incubation was continued as above, except that it occurred only in air. The samples were dialysed under running tap water; some were digested with collagenase and some used to assay hydroxy[¹⁴C]lysine, substituted hydroxy[¹⁴C]lysine and [¹⁴C]lysine by amino acid analyser. After collagenase digestion, diffusible peptides were used for the assay of hydroxy[¹⁴C]lysine. The calculations were performed as in Table 1, assuming the sum of lysine plus hydroxylysine to be 36 residues per 1000 (Miller, 1971).

	Content (residues/1000 residues)	
	Control	Temporary anoxia
Total hydroxylysine	23	14
Substituted hydroxylysine	16.0	8.4
Monosaccharide	3.8	3.3
Disaccharide	12.2	5.1
Lysine	13	22
Hydroxylysine plus lysine	36	36

These results were used to calculate the values shown in Table 2. The hydroxylysine content of the collagen synthesized under experimental conditions had fallen to about 61%, and the substituted hydroxylysine content to about 52%. Little change was found in the monosaccharide content, whereas the disaccharide content had decreased to about 42%.

Discussion

The results indicate that treatment of tendon or cartilage cells with dithiothreitol or temporary anoxia produces clear effects on the extent of the hydroxylation of the lysine residues and glycosylations of the hydroxylysine residues in the collagen synthesized, and that the effects of these two treatments are mutually opposed.

The treatment of tendon cells with dithiothreitol markedly decreased their proline hydroxylase activity, as reported previously (Berg & Prockop, 1973c; Stassen *et al.*, 1973; Tuderman *et al.*, 1975b). In accordance with this finding, a marked decrease was found in the synthesis of hydroxy[¹⁴C]proline in the cells after labelling with [¹⁴C]proline, although the rate of incorporation of [¹⁴C]proline itself only fell by about one-half. It is thus obvious that the pro- α -chains synthesized cannot form triple-helical molecules, as hydroxylation of a large number of proline residues is a requirement for triple-helix formation at 37°C (see the introduction). In addition, it is probable that the treatment with dithiothreitol prevented interchain disulphide-bond formation between the pro- α -chains (see the introduction), although this aspect was not studied here. The activities of the other intracellular enzymes of collagen biosynthesis were decreased to a lesser extent than that of proline hydroxylase; with 0.6 mM-dithiothreitol, lysine hydroxylase activity fell by about 50%, and the two collagen glycosyltransferase activities by about 10–15%.

Previous studies on the formation of hydroxylysine and glycosylated hydroxylysine in collagen have suggested that the hydroxylation of the lysine residues and glycosylations of the hydroxylysine residues must take place before triple-helix formation, as this prevents the enzyme reactions (Kivirikko *et al.*, 1973; Ryhnen & Kivirikko, 1974; Myllyl *et al.*, 1975b; Risteli *et al.*, 1976; Oikarinen *et al.*, 1976a,b). According to this theory, one would expect to find an increase in the extent of these reactions during the dithiothreitol treatment used here. The results indicate that this indeed occurred, and thus the present data give additional support for the theory. The glycosylation of hydroxylysine residues increased markedly, whereas the hydroxylation of lysine residues increased only slightly. The collagen synthesized by isolated chick-embryo tendon cells has previously been found to differ markedly from the type-I collagen isolated from adult tissues in its high degree of hydroxylation of the lysine residues (Berg & Prockop, 1973b; Barnes *et al.*, 1974; Oikarinen *et al.*, 1976b) and of the glycosylations of the hydroxylysine residues (Oikarinen *et al.*, 1976b). It has been suggested that the degree of hydroxylation of the lysine residues in this collagen may be close to the maximum value for type-I collagen (Kivirikko *et al.*, 1973; Oikarinen *et al.*, 1976b), and this may explain the failure to find any larger increase in the extent of hydroxylation of the lysine residues during treatment with dithiothreitol.

Temporary anoxia was used as a model for obtaining an accelerated triple-helix formation, as the interchain disulphide bonds are formed during the anoxia, and during the reversal period there is a rapid hydroxylation of proline residues accompanied by

triple-helix formation (see the introduction). A marked decrease was found in the extent of hydroxylation of the lysine residues in the collagen synthesized by the chick-embryo cartilage cells, a finding which is in accordance with previous data obtained with chick-embryo tendon cells (Uitto & Prockop, 1974d). An even larger decrease was found in the extent of the glycosylations of the hydroxylysine residues, the largest being in the content of glucosylgalactosyl-hydroxylysine. These results thus further support the theory concerning the effect of triple-helix formation on the extent of the post-translational modifications of the collagen polypeptide chains.

A number of reports have indicated increases in proline hydroxylase activity in various experimental and clinical conditions (see Kivirikko & Risteli, 1976; Prockop *et al.*, 1976), but it has not been demonstrated whether this enzyme activity is rate-limiting for collagen biosynthesis under normal conditions. The present results indicate that treatment of chick-embryo tendon cells with 0.1 mM-dithiothreitol decreased proline hydroxylase activity by about 75%, and the synthesis of hydroxy[^{14}C]proline in the cells during labelling with [^{14}C]proline by about 70%. Direct comparison of these two values is not possible because the rate of [^{14}C]proline incorporation decreased by about 40%, and it is not known whether this is similar with respect to collagen and other proteins. A converse complicating factor is that under normal conditions the triple helix is formed rapidly, whereas during treatment with 0.1 mM-dithiothreitol the process may be retarded and thus there may be more time for the hydroxylation of the proline residues than normal. Even with these reservations, however, the present data suggest that a close correlation exists between the proline hydroxylase activity and the synthesis of hydroxyproline. It thus seems that no very great excess of enzyme activity, at least, is present in the chick-embryo tendon cells.

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