Effect of prevention of procollagen triple-helix formation on proline 3-hydroxylation in freshly isolated chick-embryo tendon cells

Kari MAJAMAA

Department of Medical Biochemistry, University of Oulu, Kajaanintie 52A, SF-90220 Oulu 22, Finland

(Received 31 October 1980/Accepted 5 January 1981)

Inhibition of procollagen triple-helix formation by the addition of *cis*-hydroxyproline or azetidine-2-carboxylic acid increased the synthesis of 3-hydroxy[¹⁴C]proline 1.7–1.8-fold in pulse–chase experiments with freshly isolated chick-embryo tendon cells. The amount of 3-hydroxy[¹⁴C]proline, expressed as a percentage of the total ¹⁴C radioactivity in hydroxyproline, reached 8.4%. Control experiments indicated that the two analogues had no effect on the prolyl 3-hydroxylase activity of these cells. The data suggest that the time available before triple-helix formation in part regulates the extent of the 3-hydroxylation of proline in the biosynthesis of collagen in intact cells.

The hydroxyproline residues of collagen are synthesized as post-translational modifications of the procollagen polypeptide chains (Cardinale & Udenfriend, 1974; Prockop *et al.*, 1976; Kivirikko & Myllylä, 1980) in reactions catalysed by two separate enzymes, prolyl 4-hydroxylase (EC 1.14.11.2) and prolyl 3-hydroxylase (EC 1.14.11.–) (Risteli *et al.*, 1977; Tryggvason *et al.*, 1977, 1979*b*). The amount of 3-hydroxyproline in collagen is variable. Type-I and type-III collagens contain only one residue per α -chain and type-II collagen contains two residues, whereas type-IV collagens have up to about 20 residues per α -chain (Miller, 1976; Kefalides *et al.*, 1979; Kivirikko & Myllylä, 1980). This variation can be partly explained by differences in prolyl 3-hydroxylase activity between cell types (Tryggvason *et al.*, 1979*a*). It has been reported previously that prolyl 3-hydroxylase does not act on a triple-helical substrate *in vitro* (Risteli *et al.*, 1977), suggesting that the time required for triple-helix formation *in vivo* may be another factor of importance. This time varies markedly, being about 10 min in cells synthesizing type-I procollagen (Uitto & Prockop, 1973; Schofield *et al.*, 1974), 20 min in cells synthesizing type-II procollagen (Uitto & Prockop, 1974*a*; Harwood *et al.*, 1975) and 60 min in cells synthesizing basement-membrane collagen (Grant *et al.*, 1973; Williams *et al.*, 1976). After the folding of the procollagen polypeptide chains there is an additional time interval before the collagen molecule is secreted. The time of secretion varies similarly with different collagen types, being 18 min in tendon cells (Dehm & Prockop, 1972), 36 min in cartilage cells (Dehm & Prockop, 1973) and 60 min in lens cells and parietal

yolk-sac tissue (Grant *et al.*, 1972; Clark *et al.*, 1975).

The proline analogues *cis*-hydroxyproline and azetidine-2-carboxylic acid were used here to study the effect of the prevention of triple-helix formation on the 3-hydroxylation of type-I collagen in matrix-free tendon cells. These two analogues are incorporated into the procollagen polypeptide chains in place of proline (Takeuchi & Prockop, 1969; Rosenbloom & Prockop, 1971), subsequently inhibiting triple-helix formation (Uitto *et al.*, 1972; Uitto & Prockop, 1974*b*).

Experimental

Materials

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

L-Azetidine-2-carboxylic acid and *cis*-4-hydroxy-L-proline were purchased from Calbiochem (London W.1, U.K.). [¹⁴C]Proline was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.).

Isolation and incubation of matrix-free cells from tendon

Cells from 17-day chick-embryo leg tendons were isolated by using digestion with trypsin (Gibco, Grand Island, NY, U.S.A.) and bacterial collagenase (Sigma Chemical Co., Kingston-upon-Thames, U.K.) as described previously (Dehm & Prockop, 1971, 1972). The cells were filtered through a lens paper and washed three times with modified Krebs medium (Dehm & Prockop, 1971)

containing 10% (w/v) foetal-calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland, U.K.). The cell pellet was then resuspended in modified Krebs medium containing 10% (w/v) foetal-calf serum. About 10×10^6 cells were obtained from the tendons of a single embryo. The incubations were performed in a water-bath shaker at 37°C . After a 30 min preincubation with either *cis*-4-hydroxy-L-proline or L-azetidine-2-carboxylic acid, the cells were pulsed with $40 \mu\text{Ci}$ of [^{14}C]proline for 5 min and chased for 120 min. At the time points indicated, 2.0 ml portions of the incubation system (cells plus medium) were rapidly pipetted into test tubes containing 0.1 vol. of modified Krebs medium containing final concentration of $100 \mu\text{g}$ of cycloheximide/ml and $1 \mu\text{mol}$ of α -bipyridine/ml (Dehm & Prockop, 1971). The test tubes were incubated in an ice bath until all the samples had been collected. Cells plus medium were then dialysed against running tap water for 48–72 h.

Amino acid assay

The dialysed samples were hydrolysed in sealed tubes in 6M-HCl at 120°C for 20 h, and then evaporated to dryness. Next 2 ml of the buffer used in amino acid analysis was added. A portion ($50 \mu\text{l}$) was taken for the determination of total radioactivity in the sample and the rest was assayed in an amino acid analyser (Jeol 5AH amino acid analyser) for 3-hydroxyproline, 4-hydroxyproline and proline as described previously (Risteli *et al.*, 1977). Fractions (about 3.5 ml) were collected, dissolved in 5 ml of Lumagel (Lumac Systems A.G., Basel, Switzerland) and counted for radioactivity.

Assay of prolyl 3-hydroxylase activity

Three samples of freshly isolated chick-embryo tendon cells were incubated for 60 min in 9 ml of modified Krebs medium containing 10% foetal-calf serum. One sample was used as a control, and the other two were supplemented with azetidine-2-carboxylic acid and *cis*-hydroxyproline respectively to final concentrations of $100 \mu\text{g}/\text{ml}$. At the end of the incubation period the samples were centrifuged at $1200g$ for 10 min at $+4^\circ\text{C}$. The cells were then homogenized in 5 ml of a solution containing 0.2M-NaCl, 0.1M-glycine, 1mM- β -mercaptoethanol, 0.1% (w/v) Triton X-100 and 0.05M-Tris/HCl buffer adjusted to pH 7.5 at $+4^\circ\text{C}$. The homogenates were left for 1 h at $+4^\circ\text{C}$ and were then centrifuged at $15000g$ for 30 min. Portions of the supernatants were taken for the assay of prolyl 3-hydroxylase activity (Risteli *et al.*, 1978).

Other assays

The protein content of the supernatant of the cell homogenates was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

All the radioactivity counting was performed in a

Wallac liquid-scintillation spectrometer with an efficiency of 85% and a background of 25 c.p.m. for ^{14}C radioactivity, and 35% and 10 c.p.m. for ^3H radioactivity.

Fig. 1. Effect of azetidine-2-carboxylic acid on the 3-hydroxylation of [^{14}C]proline by tendon cells

Tendon cells (1.5×10^8) were incubated for 30 min in 9 ml of a modified Krebs medium containing 10% foetal calf serum, with or without the addition of $100 \mu\text{g}$ of azetidine-2-carboxylic acid/ml. The cells were then pulsed with $40 \mu\text{Ci}$ of [^{14}C]proline for 5 min, and the pulse was stopped by adding 1 ml of a modified Krebs medium containing 3 mg of unlabelled proline. At the time-points indicated, 2 ml samples of the incubation system (cells plus medium) were taken and analysed for 3-hydroxy-[^{14}C]proline, 4-hydroxy[^{14}C]proline and [^{14}C]proline. (a) Total non-diffusible 3-hydroxy-[^{14}C]proline in the cells plus medium. (b) 3-Hydroxy[^{14}C]proline as a percentage of total hydroxy[^{14}C]proline. ○, Control incubation; ●, incubation with azetidine-2-carboxylic acid.

Results

Effect of azetidine-2-carboxylic acid on the 3-hydroxylation of [14 C]proline

The 3-hydroxylation of proline in the presence of azetidine-2-carboxylic acid was studied in an experiment in which tendon cells were preincubated for 30 min with or without the analogue, pulsed with [14 C]proline for 5 min and chased with unlabelled proline for 120 min. The formation of 3-hydroxyproline proceeded similarly in the presence or absence of azetidine-2-carboxylic acid for the first 10 min of the chase period. A small additional increase was found for up to 30 min in the control cells, whereas 3-hydroxyproline formation continued for 60 min in the cells treated with azetidine-2-carboxylic acid (Fig. 1a). The total amount of non-diffusible 3-hydroxyproline was increased 1.7-fold by the addition of azetidine-2-carboxylic acid.

Expressed as a percentage of the total [14 C]radioactivity in hydroxyproline, the amount of 3-hydroxyproline reached 8.4%, whereas the corresponding value in the control cells was only 3.6% (Fig. 1b).

Effect of cis-hydroxyproline on the 3-hydroxylation of [14 C]proline

An experiment similar to that described above was carried out in an incubation medium containing 100 μ g of *cis*-hydroxyproline/ml. The total non-diffusible 3-hydroxyproline in the treated cells increased almost linearly with time during the first

60 min of the chase period, the increase during the whole chase period being about 150% in the presence of *cis*-hydroxyproline, but only about 70% in the controls (Fig. 2).

Effect of azetidine-2-carboxylic acid and cis-hydroxyproline on the activity of prolyl 3-hydroxylase

Freshly isolated tendon cells were incubated for 60 min without a proline analogue or in the presence of azetidine-2-carboxylic acid or *cis*-hydroxyproline (both 100 μ g/ml). Prolyl 3-hydroxylase activity was assayed in the 15000 g supernatant (see the Experimental section) in triplicate samples of one experiment. The mean enzyme activity was 3560 d.p.m./mg of supernatant protein in the control cells, 3130 in the cells treated with azetidine-2-carboxylic acid and 3690 in those treated with *cis*-hydroxyproline.

Discussion

The 3-hydroxylation of proline in type-I collagen, synthesized by freshly isolated chick-embryo tendon cells as their only collagen type (Uitto *et al.*, 1976), increased 1.7–1.8-fold when these cells were incubated with the proline analogues *cis*-hydroxyproline and azetidine-2-carboxylic acid, which are incorporated into the procollagen polypeptide chain in place of proline (Takeuchi & Prockop, 1969; Rosenbloom & Prockop, 1971) and lead to inhibition of triple-helix formation (Uitto *et al.*, 1972; Uitto & Prockop, 1974b). The data thus suggest that the time available before the folding of the procollagen polypeptide chains into a triple helix may in part regulate the extent of 3-hydroxylation of the procollagen in the cell.

Previous experiments have shown that incorporation of *cis*-hydroxyproline into the procollagen polypeptide chain does not affect significantly the degree of proline 4-hydroxylation or lysine hydroxylation, probably because the extents of these reactions are close to their maximum even in control cells (Rosenbloom & Prockop, 1971; Oikarinen *et al.*, 1976; Uitto *et al.*, 1978). On the other hand, the glycosylation of hydroxylysine increases by about 2.1-fold in isolated pro- γ -chains synthesized by chick-embryo tendon cells (Uitto *et al.*, 1978), and by about 1.8-fold in the collagenase-released peptides of the newly synthesized procollagen of these cells (Oikarinen *et al.*, 1976). These values are very similar to the increases in proline 3-hydroxylation found here.

The maximal 3-hydroxy[14 C]proline/total hydroxy[14 C]proline ratio was 8.4% in an experiment with azetidine, the control-cell value being 3.6%. High extents of 3-hydroxylation have previously been found in freshly trypsin-treated human fibroblasts during cell attachment. These cells secrete

Fig. 2. *Effect of cis-hydroxyproline on the 3-hydroxylation of [14 C]proline by tendon cells*

The experiment was as described in the legend to Fig. 1, but with *cis*-hydroxyproline instead of azetidine-2-carboxylic acid. O, Control incubation; ●, incubation with 100 μ g of *cis*-hydroxyproline/ml. Values are those of total radioactivity in 3-hydroxy[14 C]proline.

a substrate-attached material with a 3-hydroxyproline/4-hydroxyproline ratio of 0.051 (Lembach *et al.*, 1977) and a type-I collagen with a ratio of 0.05 (Schwartz *et al.*, 1979). When fully 4-hydroxylated tendon [^{14}C]procollagen was used as a substrate for crude prolyl 3-hydroxylase preparations, a 3-hydroxylation extent of 5.7% (7 residues per pro- α -chain) was achieved (Risteli *et al.*, 1977). Little information is available at present on the sequence requirements for interaction with prolyl 3-hydroxylase, only the minimum sequence requirement, -Gly-Pro-4Hyp-Gly-, having been established (Tryggvason *et al.*, 1977). There are about 40 such substrate sequences per α -chain in type-I collagen (Fietzek & Kühn, 1976), a value that would correspond to a maximal hydroxylation percentage of about 30%. A sequence containing *cis*-hydroxyproline or azetidine-2-carboxylic acid is probably not a substrate for the enzyme. Taking into account that non-helical procollagen synthesized under conditions similar to those used here contains about 40 residues of *cis*-hydroxyproline per α -chain (Uitto & Prockop, 1977), it can be calculated that there are about 28 intact substrate sequences in such an α -chain, allowing a maximal hydroxylation of 20%. The submaximal hydroxylation noted in the present experiments may be due in part to the secretion of a fraction of the non-helical pro- γ -chains containing the analogue (Uitto *et al.*, 1978). The interaction with prolyl 4-hydroxylase and lysyl hydroxylase is further affected by the amino acid Xaa of the triplet Xaa-Pro-Gly or Xaa-Lys-Gly, amino acids in other parts of the peptide, the peptide chain length and the peptide conformation (Kivirikko & Myllylä, 1980). The other explanation for the submaximal hydroxylation of type-I collagen here involves the possibility that not all of the 40 minimum substrate sequences -Gly-Pro-4Hyp-Gly- are accepted by the enzyme, on account of some of the factors listed above.

This work was supported in part by a grant from the Medical Research Council of the Academy of Finland. I gratefully acknowledge the valuable suggestions and comments of Professor Kari I. Kivirikko, and the expert technical assistance of Miss Helmi Konola.

References

- Cardinale, G. J. & Udenfriend, S. (1974) *Adv. Enzymol.* **41**, 245–300
- Clark, C. C., Tomich, E. A., Koszalka, T. R., Minor, R. R. & Kefalides, N. A. (1975) *J. Biol. Chem.* **250**, 5259–5267
- Dehm, P. & Prockop, D. J. (1971) *Biochim. Biophys. Acta* **240**, 358–369
- Dehm, P. & Prockop, D. J. (1972) *Biochim. Biophys. Acta* **264**, 375–382
- Dehm, P. & Prockop, D. J. (1973) *Eur. J. Biochem.* **35**, 159–166
- Fietzek, P. P. & Kühn, K. (1976) *Int. Rev. Connect. Tissue Res.* **7**, 1–60.
- Grant, M. E., Kefalides, N. A. & Prockop, D. J. (1972) *J. Biol. Chem.* **247**, 3539–3544
- Grant, M. E., Schofield, J. D., Kefalides, N. A. & Prockop, D. J. (1973) *J. Biol. Chem.* **248**, 7432–7437
- Harwood, R., Grant, M. E. & Jackson, D. S. (1975) *Biochem. J.* **148**, 129–138
- Kefalides, N. A., Alper, R. & Clark, C. C. (1979) *Int. Rev. Cytol.* **61**, 167–228
- 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
- Lembach, K. J., Branson, R. E., Hewgley, P. B. & Cunningham, L. W. (1977) *Eur. J. Biochem.* **72**, 379–383
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Miller, E. J. (1976) *Mol. Cell. Biochem.* **13**, 165–192
- Oikarinen, A., Anttinen, H. & Kivirikko, K. I. (1976) *Biochem. J.* **160**, 639–645
- Prockop, D. J., Berg, R. A., Kivirikko, K. I. & Uitto, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N. & Reddi, A. H., eds.), pp. 163–273, Plenum Press, New York
- Risteli, J., Tryggvason, K. & Kivirikko, K. I. (1977) *Eur. J. Biochem.* **73**, 485–492
- Risteli, J., Tryggvason, K. & Kivirikko, K. I. (1978) *Anal. Biochem.* **84**, 423–431
- Rosenbloom, J. & Prockop, D. J. (1971) *J. Biol. Chem.* **246**, 1549–1555
- Schofield, J. D., Uitto, J. & Prockop, D. J. (1974) *Biochemistry* **13**, 1801–1806
- Schwartz, C. E., Hellerqvist, C. G. & Cunningham, L. W. (1979) *Biochem. Biophys. Res. Commun.* **90**, 240–246
- Takeuchi, T. & Prockop, D. J. (1969) *Biochim. Biophys. Acta* **175**, 142–155
- Tryggvason, K., Risteli, J. & Kivirikko, K. I. (1977) *Biochem. Biophys. Res. Commun.* **76**, 275–281
- Tryggvason, K., Majamaa, K. & Kivirikko, K. I. (1979a) *Biochem. J.* **178**, 127–131
- Tryggvason, K., Majamaa, K., Risteli, J. & Kivirikko, K. I. (1979b) *Biochem. J.* **183**, 303–307
- Uitto, J. & Prockop, D. J. (1973) *Biochem. Biophys. Res. Commun.* **55**, 904–910
- Uitto, J. & Prockop, D. J. (1974a) *Biochemistry* **13**, 4586–4591
- Uitto, J. & Prockop, D. J. (1974b) *Biochim. Biophys. Acta* **336**, 234–251
- Uitto, J. & Prockop, D. J. (1977) *Arch. Biochem. Biophys.* **181**, 293–299
- Uitto, J., Dehm, P. & Prockop, D. J. (1972) *Biochim. Biophys. Acta* **278**, 601–605
- Uitto, J., Lichtenstein, J. R. & Bauer, E. A. (1976) *Biochemistry* **15**, 4935–4942
- Uitto, V.-J., Uitto, J., Kao, W. W.-Y. & Prockop, D. J. (1978) *Arch. Biochem. Biophys.* **185**, 214–221
- Williams, I. F., Harwood, R. & Grant, M. E. (1976) *Biochem. Biophys. Res. Commun.* **70**, 200–206