Biochem. J. (1973) **135**, 249–251
Printed in Great Britain

Evidence that Chick Tendon Procollagen Must Be Denatured to Serve as Substrate for Proline Hydroxylase

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(*Received* 15 *June* 1973)

The ability of chick-embryo proline hydroxylase to hydroxylate [^{14}C]proline-labelled procollagen was investigated between 23° and 37°C. The amount of hydroxy[^{14}C]proline that could be formed increased sharply between 26° and 30°C. This corresponded to the temperature interval in which the [^{14}C]procollagen substrate was thermally denatured, and the results therefore indicate that only denatured molecules can be hydroxylated.

The hydroxyproline in vertebrate collagens is formed from proline residues after they have been incorporated into peptide linkage in the sequence X-Pro-Gly, where X has been shown to be a number of different amino acids excluding glycine. The enzyme catalysing this hydroxylation, proline hydroxylase, has been shown to require as cofactors O_2, Fe^{2+}, 2-oxoglutarate and probably ascorbate (for reviews see Grant & Prockop, 1972*a*,*b*,*c*). Synthetic peptides containing the above sequence have also been shown to be substrates for the enzyme, and the ability of these synthetic peptides to serve as substrates appeared to increase with increasing molecular weight. However, the results are obscured somewhat by ambiguities in the conformation of some of the peptides used at the temperature of the enzymic hydroxylation (Prockop *et al.*, 1967; Kivirikko & Prockop, 1967; Hutton *et al.*, 1968). The most recent experiments, using peptides synthesized by modification of the Merrifield technique, indicated that denatured molecules were hydroxylated somewhat more readily than triple-helical ones (Kikuchi *et al.*, 1969; Kivirikko *et al.*, 1972). Other experiments, using [^{14}C]proline-labelled unhydroxylated collagen as substrate, suggested that both native and denatured molecules were about equally effective as substrates (Nordwig & Pfab, 1968; Hutton *et al.*, 1967; Kivirikko *et al.*, 1968). Rhoads *et al.* (1971) found, however, that only denatured rat tail tendon collagen could be further hydroxylated by proline hydroxylase prepared from rat skins. Recent experiments using pepsin digestion as an enzymic probe of conformation (Jimenez *et al.*, 1973) and optical methods (Berg & Prockop, 1973) have indicated that unhydroxylated collagen thermally denatures at about 24°C at acid pH. These last studies suggested that results obtained previously with [^{14}C]proline-labelled preparations of unhydroxylated collagen needed to be re-evaluated, and that significant differences could exist in the hydroxylation of such substrates depending on their conformation during the enzymic hydroxylation. The results of the present study demonstrate that chick-embryo proline hydroxylase hydroxylates only molecules of unhydroxylated chick tendon procollagen that are in the denatured state.

Materials and methods

Proline hydroxylase was purified from 13-day-old chick embryos as described by Halme *et al.* (1970). The purification was carried through the DEAE-cellulose chromatography step, and the final enzyme preparation had a specific activity of 45 units/mg of protein when measured with 125 μg of $(Pro\text{-}Gly\text{-}Pro)_n$ (molecular weight 2460)/ml as substrate (purchased from Miles Laboratories Inc., Elkhart, Indiana, U.S.A.). One unit is defined as that amount of enzyme required to synthesize 1 μg of hydroxyproline in 1 h at 37°C under conditions in which the concentrations of all other cofactors are saturating. Unhydroxylated [^{14}C]proline-labelled procollagen was prepared by incubating isolated fibroblasts in modified Krebs medium with [^{14}C]proline and 0.5 mM-$\alpha\alpha'$-bipyridyl, an Fe^{2+} chelator, for 3 h and then extracting the [^{14}C]procollagen from the cells with 0.5 M-acetic acid at 4°C (Jimenez *et al.*, 1973). The extract was centrifuged at 12000*g* for 15 min and the supernatant was then dialysed exhaustively against 0.5 M-acetic acid. This dialysed material served as substrate for the hydroxylase. About two-thirds of the label was in molecules of procollagen size (125000 daltons), as determined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, with very little in α-chains. About 70% of the procollagen was in triple-helical conformation, as determined by sensitivity to pepsin at 15°C (Jimenez *et al.*, 1973). Conditions of the hydroxylation reaction and other analytical procedures are described in Table 1 and Fig. 1.

Results

On the basis of previous experiments (Jimenez *et al.*, 1973; Berg & Prockop, 1973) we estimated the denaturation temperature of unhydroxylated collagen to be about 26–27°C at neutral pH. Since we wished to study the effect of substrate conformation on the extent of hydroxylation while minimizing the usual effect of temperature on enzymic reactions, detailed experiments were first carried out at 24° and 30°C. At 24°C we would expect the molecules to be triple-helical, whereas at 30°C they would be denatured. When 27000d.p.m. of the unhydroxylated [^{14}C]-procollagen preparation was incubated for from 1h to 4h at 24°C with 4 units of proline hydroxylase about 2600d.p.m. of hydroxy[^{14}C]proline was formed, independent of the incubation time. Doubling the amount of enzyme initially present did not affect the extent of hydroxy[^{14}C]proline formation, nor did the addition of more enzyme during the course of the incubation (Table 1). Preincubation of the substrate for 1h before the addition of the enzyme and cofactors did not alter the amount of hydroxyproline formed. When the incubation was carried out at 30°C, slightly more than 7600d.p.m. of hydroxy[^{14}C]proline was formed, a value that was also independent of the incubation time. Here again, as demonstrated above, doubling the amount of enzyme initially present or addition of fresh enzyme did not affect the extent of hydroxylation. Thus a 6°C change in temperature approximately tripled the amount of hydroxy[^{14}C]proline that could be formed in the reaction.

In order to study the temperature-dependence in greater detail, portions of the unhydroxylated [^{14}C]-procollagen were incubated with proline hydroxylase over the temperature range 23–37°C. A 2h incubation time was chosen, since the reaction appeared completed by then. The results shown in Fig. 1 demonstrate that the amount of hydroxyproline formed remained nearly constant up to 26°C, at about 9% of the total ^{14}C in the incubation. Above 26°C there was a sharp increase, until a maximum of about 28% of the radioactivity was found in hydroxy[^{14}C]proline at 30°C and higher temperatures.

Table 1. *Hydroxylation of* [^{14}C]*procollagen at* 24° *and* 30°C

Each 1ml of reaction mixture contained 27000d.p.m. of unhydroxylated [^{14}C]procollagen, proline hydroxylase as indicated, 1mg of bovine serum albumin and 0.05mg of catalase, in addition to 0.05mM-$FeSO_4$, 0.5mM-2-oxoglutarate, 0.1mM-dithiothreitol and 50mM-Tris–HCl buffer, pH7.8. The values represent the average of duplicate incubations of 1ml volumes each. The incubation was terminated by the addition of 1ml of 12M-HCl and the mixture was then hydrolysed at 108°C for 24h. The hydroxy[^{14}C]proline content of the samples was then assayed by a specific chemical procedure (Juva & Prockop, 1966).

Incubation condition	Incubation time (h)	Hydroxy[^{14}C]proline (d.p.m.)	Degree of hydroxylation* (%)
Incubation at 24°C			
No enzyme	2	<50	<0.2
4 units of enzyme	1	2523	9.3
4 units of enzyme	2	2535	9.4
4 units of enzyme	4	2833	10.5
8 units of enzyme	2	2473	9.2
4 units of enzyme from zero time, then 4 units added at 1h	2	2715	10.1
Substrate preincubated for 1h, then added to mixture with 4 units of enzyme	2	2580	9.6
Incubation at 30°C			
No enzyme	2	<50	<0.2
4 units of enzyme	1	7735	28.7
4 units of enzyme	2	7306	27.1
4 units of enzyme	4	7590	28.1
8 units of enzyme	2	7344	27.2
Substrate preincubated for 1h, then added to mixture with 4 units of enzyme	2	8093	30.0

* Values are 100 times hydroxy[^{14}C]proline per total ^{14}C.

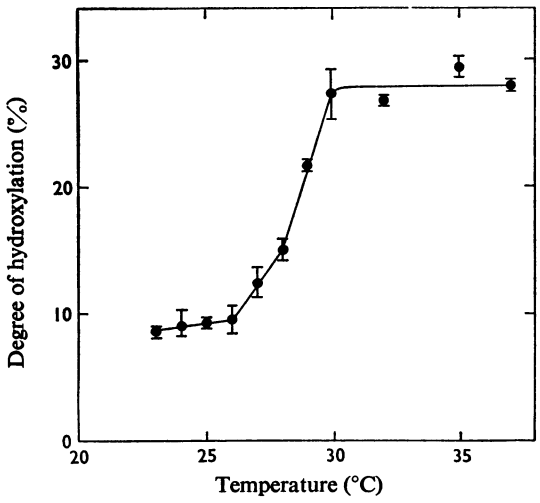

Fig. 1. *Hydroxylation of [^{14}C]procollagen at various temperatures for 2h*

Reaction mixtures (1 ml) containing 4 units of proline hydroxylase and 27000d.p.m. of unhydroxylated [^{14}C]procollagen were used. Other conditions and analytical procedures are described in Table 1. Each point represents the average of at least duplicate incubations. The bars indicate the ranges found in the determinations at each temperature.

Discussion

Previous experiments designed to determine the effect of conformation on the hydroxylation of biologically labelled unhydroxylated collagen (Nordwig & Pfab, 1968; Hutton *et al.*, 1967; Kivirikko *et al.*, 1968) need to be reinterpreted, since the substrates thought to be triple-helical were probably denatured at the temperature of the enzymic incubation, usually 37°C. The present results suggest that only denatured molecules (or possibly regions of local denaturation) can be hydroxylated. The basal value of 9% hydroxyproline content formed at temperatures below 26°C is probably due to the constant presence of denatured procollagen chains in the substrate as it was prepared. It is likely that the unhydroxylated [^{14}C]procollagen is denatured within the cell at 37°C and forms triple-helical molecules during the extraction and dialysis procedure (Jimenez *et al.*, 1973; Berg & Prockop, 1973). The acetic acid extracts contain 10–35% of the procollagen in molecules that are not triple-helical, as determined by sensitivity to pepsin at 15°C. The particular preparation used in the present work contained about 30% of denatured chains. When the temperature of the enzymic incubation approached the denaturation temperature of the substrate, the quantity of hydroxy[^{14}C]proline synthesized increased sharply over a 4°C range (26–30°C) and reached a maximal degree of hydroxylation of 28%. Since only about two-thirds of the label was in procollagen and since fully hydroxylated procollagen has a degree of hydroxylation of about 44%, the value of 28% probably represents close to the theoretical maximum. Under the conditions of the assay, the degrees of hydroxylation obtained at the temperatures between 26° and 30°C appear to represent maximal values for the particular temperature. It is possible that the incomplete hydroxylation obtained at the intermediate temperatures may be caused by the formation of triple-helical molecules by the partially hydroxylated chains. The present experiments suggest that the hydroxyproline content of the collagen in different animals may in part be governed by the body temperature and not entirely by the primary amino acid sequence that is encoded genetically.

This work was supported by National Institutes of Health Research Grants AM-14439, AM-14526 and RR-107, the National Institute of Dental Research U.S. Public Health Service Grant DE-02623 and the American Heart Association Grant 70717.

Berg, R. & Prockop, D. J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 115–120
Grant, M. E. & Prockop, D. J. (1972*a*) *N. Engl. J. Med.* **286**, 194–199
Grant, M. E. & Prockop, D. J. (1972*b*) *N. Engl. J. Med.* **286**, 242–249
Grant, M. E. & Prockop, D. J. (1972*c*) *N. Engl. J. Med.* **286**, 291–300
Halme, J., Kivirikko, K. I. & Simons, K. (1970) *Biochim. Biophys. Acta* **198**, 460–470
Hutton, J. J., Tappel, A. L. & Udenfriend, S. (1967) *Arch. Biochem. Biophys.* **118**, 231–240
Hutton, J. J., Marglin, A., Witkop, B., Kurtz, J., Berger, A. & Udenfriend, S. (1968) *Arch. Biochem. Biophys.* **125**, 779–785
Jimenez, S., Harsch, M. & Rosenbloom, J. (1973) *Biochem. Biophys. Res. Commun.* **52**, 106–114
Juva, K. & Prockop, D. J. (1966) *Anal. Biochem.* **15**, 77–83
Kikuchi, Y., Fujimoto, D. & Tamiya, N .(1969) *Biochem. J.* **115**, 569–574
Kivirikko, K. I. & Prockop, D. J. (1967) *J. Biol. Chem.* **242**, 4007–4012
Kivirikko, K. I., Bright, H. J. & Prockop, D. J. (1968) *Biochim. Biophys. Acta* **151**, 558–567
Kivirikko, K. I., Kishida, Y., Sakakibara, S. & Prockop, D. J. (1972) *Biochim. Biophys. Acta* **271**, 347–356
Nordwig, A. & Pfab, F. K. (1968) *Biochim. Biophys. Acta* **154**, 603–605
Prockop, D. J., Juva, K. & Engel, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 553–560
Rhoads, R. E., Udenfriend, S. & Bornstein, P. (1971) *J. Biol. Chem.* **246**, 4138–4142