SEPARATION OF PROLYL 3-HYDROXYLASE AND 4-HYDROXYLASE ACTIVITIES AND THE 4-HYDROXYPROLINE REQUIREMENT FOR SYNTHESIS OF 3-HYDROXYPROLINE

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Received April 1,1977

SUMMARY: Differences between prolyl 3-hydroxylase and prolyl 4-hydroxylase activities were found in their stimulation and inactivation by dithiothreitol and in their affinity to poly-L-proline linked to agarose. The two enzyme activities were separated by gel filtration, the results demonstrating that they are due to separate proteins. Comparison of [^{14}C]proline-labelled protocollagen and the same protein when fully 4-hydroxylated as substrates indicated dependence of 3-hydroxyproline formation on the presence of 4-hydroxyproline. It is suggested that the main substrate sequence for 3-hydroxyproline synthesis is -Gly-Pro-4Hyp-Gly-.

Hydroxyproline is found in the tissues of vertebrates almost exclusively in collagen. Most of this imino acid is present as the trans-4-isomer, but all collagens contain some trans-3-hydroxyproline (1-3). Prolyl hydroxylase, which catalyses the formation of 4-hydroxyproline, has been isolated as a pure protein from several sources and extensively characterized (for reviews, see 1,4,5). No synthesis of 3-hydroxyproline was detected with pure prolyl 4-hydroxylase from chick embryos, suggesting that separate enzymes may be involved in the synthesis of the two isomers (6,7). The formation of 3-hydroxyproline in vitro has been studied recently with a crude rat kidney cortex extract as the enzyme source (7). Many properties of this reaction, such as its co-substrate and co-factor requirements, the preferential hydroxylation of longer polypeptide chains and the non-helical substrate requirement, were found to be similar to those of the 4-hydroxylase reaction (7). However, the 3-hydroxylase reaction was not inhibited by antiserum to prolyl 4-hydroxylase or poly-L-proline when studied with chick tendon procollagen as a substrate, these data further suggesting that prolyl 3-hydroxylase and 4-hydroxylase

ISSN 0006-291X

are separate enzymes (7). The present paper reports the separation of these two enzyme activities.

The only 3-hydroxyproline residue in type I collagen is found in a sequence -Gly-3Hyp-4Hyp-Gly- (2,3,8), and most, or perhaps all, in type IV collagen, which contains 10-15 residues of this imino acid per α chain, is likewise in this sequence (9). The main substrate sequence for 3-hydroxyproline synthesis must therefore be either -Gly-Pro-4Hyp-Gly- or -Gly-Pro-Pro-Gly-. It has previously been suggested that the former might be the substrate sequence (7). In the present work this aspect is studied further by comparing the hydroxylation of chick tendon protocollagen, which contains no 4-hydroxyproline, and the same protein in which all appropriate prolyl residues had been converted to 4-hydroxyproline by incubation with a large excess of pure chick prolyl 4-hydroxylase *in vitro*.

MATERIALS AND METHODS: Kidney cortex was excised from 2-month-old Sprague-Dawley rats, immediately frozen in liquid nitrogen and stored at -70°C until used. The cortex was homogenized in a Teflon and glass homogenizer in a cold solution of 0.2 M NaCl, 0.1 M glycine, 50 μM dithiothreitol, 0.1 % (w/v) Triton X-100, 0.01 % (w/v) soybean trypsin inhibitor and 20 mM Tris-HCl buffer adjusted to pH 7.5 at 4°C, using 4 ml/g kidney cortex. The homogenate was incubated at 4°C for 20 min and centrifuged at 15,000 x g for 30 min at 4°C (7). A new enzyme extract was prepared for each experiment.

Tendon protocollagen was prepared in isolated cells obtained from the leg tendons of 60 17-day-old chick embryos as reported previously (7,10,11). The final preparation was heated to 100°C for 10 min, centrifuged at 1,000 x g for 10 min to remove the precipitate formed during heating, and incubated with 5 μg of pure chick prolyl 4-hydroxylase (12) for 4 h at 37°C in a standard incubation mixture (1,5,12), but omitting bovine serum albumin and catalase, to convert all appropriate prolyl residues to 4-hydroxyprolyl residues. The reaction was stopped by heating to 100°C for 5 min, the solution dialysed against 0.2 M NaCl and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 4°C, and the preparation stored frozen in aliquots. This constituted the standard substrate. About 29 % of the [^{14}C]prolyl residues in this substrate were found as 4-hydroxy[^{14}C]proline, whereas no 3-hydroxy[^{14}C]proline was detectable using an amino acid analyzer (7). The percentage of 4-hydroxy-[^{14}C]proline was lower than that in procollagen, since the preparation used here was not pure. In the experiment to study the hydroxylation of protocollagen a portion of the same substrate was used without treatment with prolyl 4-hydroxylase.

The prolyl 3-hydroxylase reaction under standard conditions was carried out for 40 min at 20°C in a final volume of 2.0 ml containing 100,000 dpm [^{14}C]proline-labelled substrate, 0.08 mM $FeSO_4$, 2 mM ascorbic acid, 0.5 mM 2-oxo-glutarate, 0.2 mg/ml catalase, 2 mg/ml bovine serum albumin, 0.1 mM dithiothreitol and 50 mM Tris-HCl buffer adjusted to pH 7.8 at 25°C (7). The reaction

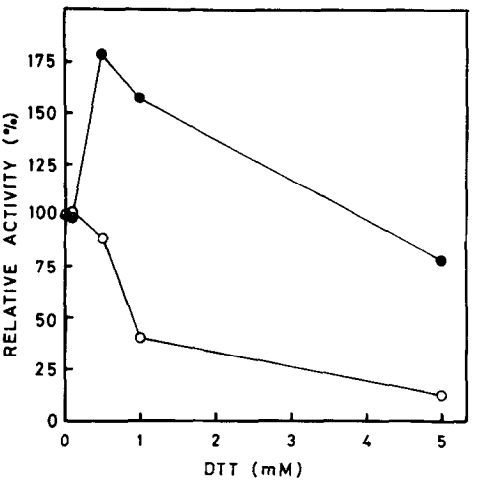

Fig. 1. Effect of preincubation with varying concentrations of dithiothreitol on prolyl 3-hydroxylase and 4-hydroxylase activities. The supernatant of rat kidney cortex homogenate was preincubated with varying concentrations of dithiothreitol at 20°C for 1 h, and prolyl 3-hydroxylase (●) and 4-hydroxylase (o) activities were then measured. Both enzyme activities are expressed as percentages of the value observed in a sample preincubated without dithiothreitol.

was stopped by adding an equal volume of concentrated HCl, and the samples hydrolysed and assayed using an amino acid analyzer (7). The prolyl 4-hydroxylase reaction was performed with 50,000 dpm [^{14}C]proline-labelled protocollagen as substrate (12).

RESULTS: Attempts were made to determine whether there are any further differences between prolyl 3-hydroxylase and 4-hydroxylase activities. A clear difference between the two enzyme activities was found after preincubation with varying concentrations of dithiothreitol at 20°C for 1 h. This compound is known to stimulate pure prolyl 4-hydroxylase in low concentrations, whereas in higher concentrations it dissociates the enzyme into inactive subunits (see 1,5). Essentially no stimulation is usually to be found with crude enzyme preparations, however (1,5). The present experiments indicated stimulation of prolyl 3-hydroxylase activity even with 0.5 and 1.0 mM dithiothreitol, while the latter concentration in particular was a powerful inhibitor of the 4-hydroxylase activity (Fig. 1).

Several procedures were tested for the separation of the two enzyme activities. Both enzymes were found to be acidic proteins in that they became bound to DEAE-cellulose with a 0.25 M NaCl

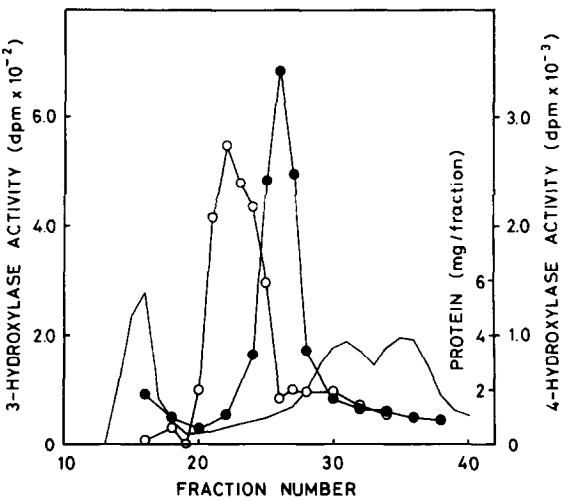

Fig. 2. Separation of prolyl 3-hydroxylase and 4-hydroxylase activities by gel filtration on an 8 % agarose column. Two ml of the supernatant of rat kidney cortex homogenate was applied to a Bio-Gel A-1.5 m column (200-400 mesh, Bio-Rad), 1.5 x 90 cm, which was equilibrated and eluted at 4°C with a solution consisting of 0.1 M NaCl, 0.1 M glycine and 20 mM Tris-HCl buffer adjusted to pH 7.5 at 4°C. Fractions of 2.5 ml were collected. The prolyl 3-hydroxylase activity (●) was assayed in 0.7 ml of the fraction and expressed per 100,000 dpm of the 4-hydroxylated substrate (7), and the prolyl 4-hydroxylase activity (o) in 0.1 ml with 50,000 dpm of the protocollagen substrate (12). The amount of protein in the fractions is indicated by the continuous line.

concentration at pH 7.5, under conditions in which most other proteins are eluted. Both enzymes could be eluted completely with 0.4 M NaCl, and thus seem to behave rather similarly in respect of this separation technique.

Prolyl 4-hydroxylase activity can be effectively purified by affinity chromatography on poly-L-proline linked to agarose (12). This technique gave partial but not complete separation of the two enzyme activities. In experiments with a 3-ml affinity column, the 4-hydroxylase activity became essentially completely bound initially, while the 3-hydroxylase activity was partially bound. When 30 ml of enzyme extract containing 20 mg/ml protein was passed through the column, the 4-hydroxylase activity per ml of column effluent increased to about 15 % of that in the sample applied to the column and the 3-hydroxylase activity to about 60 %.

Complete separation of the two enzyme activities was obtained by gel filtration on an 8 % agarose column (Fig. 2). Here the

TABLE 1. Substrate 4-hydroxyproline requirement for the synthesis of 3-hydroxyproline

Substrate	Condition	3Hyp (dpm)	4Hyp (dpm)
4Hyp-free	- Enzyme	<50	140
	+ Enzyme	<50	1,530
4Hyp present	- Enzyme	<50	28,900
	+ Enzyme	390	28,600

[^{14}C]Proline-labelled protocollagen and fully 4-hydroxylated protein were compared as substrates for the synthesis of 3-hydroxyproline, as described in Methods. The gel filtration fractions containing little 4-hydroxylase activity were pooled, and 0.7 ml (1.1 mg protein) of the pool used as the source for the 3-hydroxylase. After hydrolysis, the products were assayed using an amino acid analyzer (7). The values are given as dpm per 100,000 total dpm.

prolyl 3-hydroxylase activity was eluted later than that of 4-hydroxylase, and no significant activity of the former enzyme was found in the peak fraction of that of the latter, or *vice versa*. Calibration of the gel filtration column with standard proteins suggested a molecular weight of about 160,000 daltons for the 3-hydroxylase, provided that it is a normal globular protein.

In order to study the requirement for 4-hydroxyproline in the polypeptide substrate for the synthesis of 3-hydroxyproline, [^{14}C]-proline-labelled protocollagen and fully 4-hydroxylated protein were compared as substrates, as described in Methods and in Table 1. The gel filtration fractions containing little 4-hydroxylase activity were pooled and used as the source of 3-hydroxylase in this experiment to avoid any significant formation of 4-hydroxyproline in the 4-hydroxyproline-free substrate during incubation. No synthesis of 3-hydroxy[^{14}C]proline was detected in the 4-hydroxy-[^{14}C]proline-free substrate, although some 4-hydroxy[^{14}C]proline was synthesized during the incubation (Table 1). By contrast, a definite synthesis of 3-hydroxy[^{14}C]proline took place in the maximally 4-hydroxylated substrate, thus indicating a clear difference in the 3-hydroxylation of these two proteins.

DISCUSSION: Previous studies have suggested that prolyl 3-hydroxylase and 4-hydroxylase are separate enzymes, since the 3-hydroxyproline formation in chick tendon procollagen is not inhibited by antiserum to prolyl 4-hydroxylase or by poly-L-proline in concentrations which clearly inhibit 4-hydroxyproline formation in chick tendon protocollagen (7), and since pure prolyl 4-hydroxylase synthesizes no 3-hydroxyproline (6,7). The present results indicate additional differences between these enzyme activities in their stimulation and inactivation by dithiothreitol and in their affinity to columns of poly-L-proline linked to agarose. Final proof that these two enzyme activities originate from separate proteins was obtained by their separation in gel filtration. Accordingly prolyl 3-hydroxylase constitutes a third hydroxylase involved in collagen biosynthesis, in addition to prolyl 4-hydroxylase and lysyl hydroxylase.

Comparison of [^{14}C]proline-labelled protocollagen and the same protein when fully 4-hydroxylated as substrates indicated a clear dependence of 3-hydroxyproline formation on the presence of 4-hydroxyproline in the substrate. As the sequence in which 3-hydroxyproline has been identified in vertebrate collagens is -Gly-3Hyp-4Hyp-Gly- (2,3,8,9), the results suggest that the main substrate sequence for 3-hydroxyproline synthesis is -Gly-Pro-4Hyp-Gly-.

It has recently been reported that the 3-hydroxylase does not catalyze the reaction with a triple-helical substrate (7). This finding and the present results suggest that 3-hydroxyproline is formed in collagen biosynthesis after the 4-hydroxylation of an appropriate number of prolyl residues, but before the triple helix formation. On the basis of data reported for the rates of 4-hydroxyproline formation and the triple helix formation in cells synthesizing type I or type IV collagen, it can be estimated that the time available for 3-hydroxyproline formation in the biosynthesis of type IV collagen may be up to 10 times that in type I collagen (13-16). Accordingly, the 10-fold difference in 3-hydroxyproline content between these two collagens (2,3), may be attributable to the time factor, although naturally the present results do not exclude the possibility that there are also differences in the levels of 3-hydroxylase activity between the cells synthesizing these two types of collagen.

ACKNOWLEDGEMENTS: The work was supported in part by a grant from the Medical Research Council of the Academy of Finland. We thank Miss Helmi Konola for her expert technical assistance.

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