STUDIES ON THE STABILITY OF PROTOCOLLAGEN HYDROXYLASE

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1. Introduction

The enzyme protocollagen hydroxylase [1,2] synthesises hydroxyproline by the hydroxylation of proline in protocollagen, the large proline-rich and lysine-rich polypeptide precursor of collagen (for review, see [3,4]). The enzyme does not hydroxylate free proline, proline in tripeptides, or proline in poly-L-proline [5], but hydroxylates proline in polytripeptides of the structure $(L\text{-Pro-Gly-L-Pro})_n$ or $(Gly\text{-L-Ala-L-Pro})_n$ [1,2, 5-7]. Procedures for the partial purification of protocollagen hydroxylase have been reported [1,5,7]; however, the losses of enzyme have been relatively large, and the most highly purified preparations of the enzyme have rapidly lost their activity. As a result, it has been impracticable to work with these preparations, and studies of the characterisation of protocollagen hydroxylase have been made with enzyme preparations of a lower degree of purification [1,2,7]. In the present study, it was found that the addition of glycine protects protocollagen hydroxylase from losses of activity; the enzyme retained the highest degree of activity in solutions containing glycine and with pH values around 8. By the application of these conditions, it proved possible to develop a modified procedure for obtaining more purified preparations of the enzyme.

2. Methods

The protocollagen hydroxylase was prepared from extracts of 12-day-old chick embryos, as has been described earlier [1, 2, 7], with the following modifications. The solutions used in dialysis at various stages of the purification procedure contained 0.05 M KCl, 0.1 M NaCl, 0.1 M glycine, and 0.01 M tris-HCl buffer, pH 7.8 at +4°. In the calcium phosphate gel step [7], a gel containing 15 mg solid/ml was used, and the amount of gel utilised to obtain the pellet fractions was increased to 6.5 ml per 1000 mg protein. Only three pellets were prepared; these were eluted with 0.02 M and 0.15 M potassium phosphate, as described earlier [7], but the pH of the solutions was increased to 7.8, and 0.1 M glycine was added. In the subsequent ammonium sulphate precipitation, the amount of ammonium sulphate was raised to 55% saturation. Before experiments were made on the stability of protocollagen hydroxylase, glycine was removed from the enzyme preparation by dialysis against 0.05 M KCl and 0.01 M tris-HCl buffer, pH 7.8.

The enzyme reaction under standard conditions was effected in a final volume of 4 ml, containing 0.05-0.3 mg/ml enzyme protein; 125 μg/ml $(Pro\text{-}Gly\text{-}Pro)_n$, M. W. 6600; 0.08 mM $FeSO_4$; 0.5 mM α-ketoglutarate; 2 mM ascorbic acid; 0.05 mg/ml catalase, and 0.05 M tris-HCl buffer, adjusted to pH 7.8 at 25° [2]. In addition, 0.1 mM dithiothreitol (DTT) was used in most experiments [8]. The samples were incubated at 37° for 1 h, as described earlier [2], and after hydrolysis, the amount of hydroxyproline synthesised was assayed [9]. The protein content of the enzyme preparations was assayed by peptide absorbance at 225 nm, and by ninhydrin assays of protein hydrolysates.

3. Results and discussion

For the study of a possible stabilising effect of

Table 1

Synthesis of hydroxyproline by protocollagen hydroxylase preparations dialysed against various solutions for 48 h at +4° *

Dialysis solution	Hydroxy-proline formed (μg)	% of non-dialysed control
Experiment I		
No dialysis †	10.8	100
Asc. + Fe^{++} + αKG **	5.0	46
DTT, 0.1 mM	7.3	68
Mercaptoethanol, 0.1 mM	6.9	64
Glycine, 0.1 M	9.9	92
Experiment II		
No dialysis †	16.0	100
DTT, 0.1 mM	9.9	62
Glycine, 0.1 M	15.2	95
Proline, 0.01 M	6.9	43
Alanine, 0.01 M	7.4	46
Experiment III		
No dialysis †	11.0	100
Glycine, 0.1 M	10.8	98
Glycine, 0.05 M	10.3	94
Glycine, 0.01 M	9.5	86
Glycine, 0.001 M	5.1	46
No glycine	3.7	34

* All the solutions contained 0.05 M KCl, and 0.01 M tris-HCl buffer, pH 7.8 at +4°. After dialysis, 0.3 mg/ml of the enzyme protein was tested in the hydroxylation reaction under standard conditions with 0.1 mM DTT.

** Ascorbate, 0.1 mM; Fe^{++}, 0.05 mM; α-ketoglutarate, 0.1 mM.

† Stored frozen.

various compounds on protocollagen hydroxylase, preparations of the enzyme were dialysed at +4° for 48 h against various solutions, and the activities of the enzyme preparations were determined. The hydroxylation reaction with protocollagen hydroxylase requires atmospheric oxygen, ferrous iron, α-ketoglutarate, and ascorbate (cf. [3, 4]), but attempts to employ the three last-mentioned substances alone, or in various combinations, as stabilisers of the enzyme were unsuccessful (table 1). Moreover, no reproducible protection of the enzyme resulted when DTT, or mercaptoethanol, was added in various concentrations to the dialysis solutions. The polypeptide substrate could not be tested, as it was available only in small quantities; however, effect of the amino acids glycine and proline was studied. Reproducible protection of the enzyme was gained with 0.05 M or 0.1 M glycine, whereas proline, alanine or the dipeptide glycyl-glycine was not effective. It may be noted that glycine occupies every third position in the polypeptides which can be used as substrates for the enzyme, but the mechanism involved in the protecting effect of glycine is unknown.

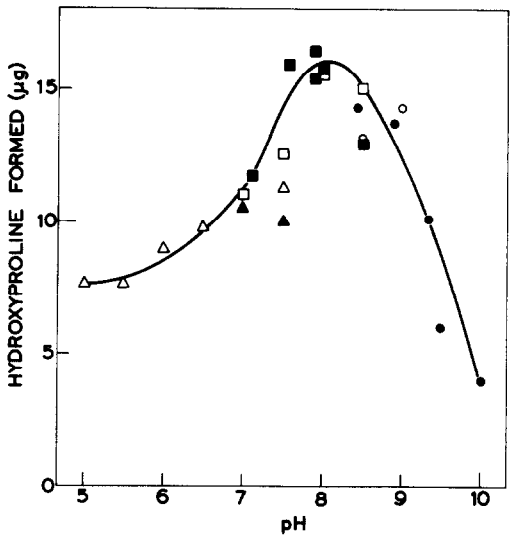

Fig. 1. Synthesis of hydroxyproline by protocollagen hydroxylase preparations dialysed against buffers at various pH values for 48 h at +4°. Dialysis solutions: 0.01 M sodium phosphate buffer containing 0.1 M glycine and 0.1 M NaCl (△, ▲); 0.01 M tris-HCl buffer containing 0.1 M glycine and 0.1 M NaCl (□, ■); 0.1 M glycine-NaCl buffer (○, ●). Open symbols, experiment I; closed symbols, experiment II. All values in experiment I were multipled by a factor 1.6 for comparison with the values of experiment II.

Previously, the pH optimum of the enzyme in the hydroxylation reaction was reported as from 7.3 to 7.4 at 37° [2]. In the present study, the effect of pH on the stability of the enzyme during 48-h dialysis was studied. It was found that the enzyme retained its highest degree of activity in glycine-containing buffers with a pH value around 8. Dialysis in pH values below 7.5 resulted in considerable losses of the activity (fig. 1).

The addition of small amounts of catalase to the incubation mixture protects protocollagen hydroxylase from losses of activity during the hydroxylation reaction [2]. A stimulatory effect of DTT and bovine

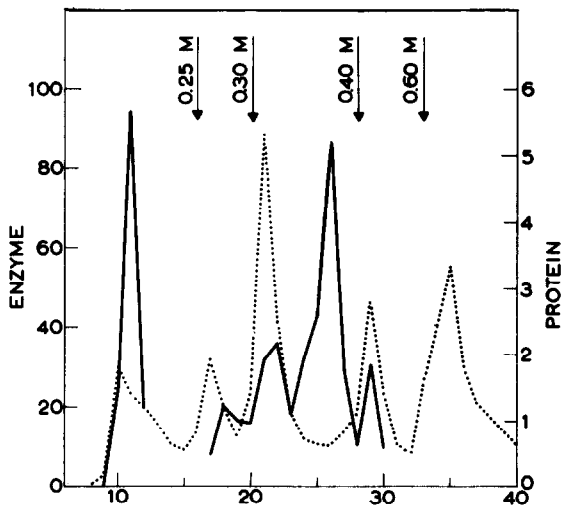

Fig. 2. Chromatography of protocollagen hydroxylase purified through the calcium phosphate gel step on a DEAE cellulose column, 1.5 X 24 cm by size. The column was equilibrated with a solution containing 0.2 M NaCl, 0.2 M glycine, and 0.01 M tris-HCl buffer, pH 7.0 at +4°, and was eluted step-wise with solutions containing 0.20 M, 0.25 M, 0.30 M, 0.40 M and 0.60 M NaCl and glycine, and 0.01 M tris-HCl buffer, pH 7.0. In the experiment shown, 45 mg of enzyme protein synthesising 15.4 μg hydroxyproline per mg protein was applied to the column, and 4-ml fractions were collected in test tubes containing 1.0 ml 0.2 M glycine-NaCl buffer, pH 8.5. Broken line: protein (mg/fraction); solid line: enzyme activity (μg hydroxyproline synthesised/fraction).

serum albumin (BSA) on the enzyme during the hydroxylation reaction has recently been described by Rhoads et al. [8], in an incubation system in which catalase was not used. In the present study, when either DTT or BSA was added to the incubation mixture containing catalase, a small stimulation ranging from 5 to 20 per cent was observable, although no additional stimulation was remarked on the combined use of catalase, DTT and BSA. When glycine was removed from the enzyme preparation by dialysis, and 100 to 200 μl of 0.1 M glycine was added to the 4-ml incubation mixture, no changes were discernible in the enzyme activity. This amount of glycine corresponds to the amounts added with the enzyme preparations, and the results suggest that the protection of the enzyme by glycine is not attributable to protection during the hydroxylation reaction.

The chick embryo extracts synthesised 0.03 to 0.1 μg hydroxyproline-^{14}C per mg protein per h, when 125 μg/ml of proline-^{14}C labelled $(Pro\text{-}Gly\text{-}Pro)_n$ was used as a substrate [6]. The best enzyme preparations obtained previously after the calcium phosphate gel step synthesised 9-13 μg hydroxyproline per mg protein per h, when 125 μg/ml of $(Pro\text{-}Gly\text{-}Pro)_n$ was used as substrate [7]. The corresponding preparations obtained by the present procedure synthesised up to 22 μg hydroxyproline per mg protein per h with a recovery of about 40 per cent of the total activity present in the ammonium sulphate fraction [2]. To bring about further purification of the enzyme, chromatography on a DEAE cellulose column was attempted. Efforts to purify the enzyme by chromatography at pH values around 8 proved unsuccessful, and accordingly chromatography at pH 7.0 was applied (fig. 2). To avoid losses of enzyme activity, glycine was added to all the solutions, and the fractions were collected in test tubes containing 0.2 M glycine-NaCl buffer at pH 8.5. The best fraction obtained synthesised 140 μg hydroxyproline per mg protein per h, and when fractions nos. 11, 24-27 were pooled (fig. 2), an enzyme preparation synthesising 68 μg hydroxyproline per mg protein per h and containing 41 per cent of the total activity applied to the column was obtained. In another similar chromatography, there was obtained a preparation synthesising 62 μg hydroxyproline per mg per h with a recovery of 34 per cent. These preparations retained about 90 per cent of their activity after storage for 2 weeks at -20°.

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