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XVII. PROTOCOLLAGEN HYDROXYLASE OF PIG UTERUS

KUNG-YING TANG KAO, C. R. TREADWELL, J. M. PREVILL* AND THOMAS H. McGAVACK

Geriatrics Research Laboratory, Veterans Administration Center, Martinsburg, W. Va. and Departments of Biochemistry and Medicine, George Washington University School of Medicine, Washington, D.C. (U.S.A.)

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SUMMARY

A highly active protocollagen hydroxylase, comparable to that from chick embryos, was found in homogenate of porcine uterus. For maximum activity, atmospheric O_2, Fe^{2+}, asorbic acid and α-ketoglutarates are required. The uterine hydroxylase catalyzes the hydroxylation of both proline and lysine in protocollagen prepared from bone of chick embryos. The uterine tissue could be stored at $-30°$ for several weeks without a decrease in enzymic activity.

INTRODUCTION

A cell-free system prepared from chick embryos for studying collagen biosynthesis has been reported by PETERSKOFSKY AND UDENFRIEND[1,2]. Later, PROCKOP, WEINSTEIN AND MULVENY[3] described the use of α,α'-dipyridyl as a chelating agent for iron to inhibit the hydroxylation of proline in embryonic tibiae. This allowed the preparation of the collagen precursor, protocollagen, which then could be utilized as a substrate for the hydroxylation by the protocollagen hydroxylase prepared from chick embryo. With this system a good level of hydroxylation of the protocollagen prepared with either [^{14}C]proline (see ref. 4) or [^{14}C]lysine (see ref. 3) has been obtained. The hydroxylating enzyme has been shown to be present also in skin, liver and granuloma tissues of adult animals[5]. However, the co-factor requirements for the hydroxylating system have not been determined for these tissues. In earlier work, we have reported that the uterine tissue of rat is highly active in the biosynthesis of collagen[6,7]. As a part of our long-term studies in changes in uterine tissue with age[7] and stages of the estrus cycle[8–10], we have investigated the occurrence and characteristics of protocollagen hydroxylase in homogenates of pig uterus.

* Present address: West Virginia University School of Medicine, Morgantown, W.Va., U.S.A.

METHODS

Preparation of enzyme extract. Uteri of pigs, 6 months to 3 years of age, were obtained from a local slaughter house. The chilled tissue was cut into small pieces and passed through a hand meat grinder. The ground tissue was homogenized with an equal volume of 0.25 M sucrose* in a VirTis "45" homogenizer at its lowest speed for 30 sec. The homogenate was centrifuged at 15 000 × *g* for 15 min and the precipitate was discarded. The supernatant fluid which contained 20–30 mg protein per ml was used as the crude enzyme extract.

Preparation of protocollagen substrate. The tibiae and femurs of 9 chick embryos (11 days old) were incubated in 5 ml Krebs–Henseleit solution[11] containing $1 \cdot 10^{-3}$ M α,α'-dipyridyl for 45 min at 37°. This initial incubation chelated Fe^{2+} normally required for the hydroxylation of the labeled protocollagen to be formed in the next step. 6 μC of [^{14}C]proline (Schwarz BioResearch, Inc.) were then added and the mixture incubated under 95% O_2–5% CO_2 at 37° for an additional 2 h. During this period a proline-rich and hydroxyproline-poor protocollagen was formed. The reaction was terminated by lowering the temperature to −28°. The tibiae and femurs were removed, washed 3 times with distilled water, cut into small pieces and homogenized in 16 ml distilled water. The homogenate was centrifuged at 100 000 × *g* for 1 h. The supernatant was dialyzed against distilled water overnight and used as the source of substrate. Each ml contained approx. 0.1 mg of protein[12] and 20 000–100 000 disint./min per mg of protein.

Co-factors. A solution was prepared so that the known co-factors were present at 10 times the concentration desired in the final incubation system, *i.e.* ascorbic acid, $1 \cdot 10^{-3}$ M; $FeSO_4 \cdot 6\ H_2O$, $0.2 \cdot 10^{-3}$ M; α-ketoglutarate, $0.2 \cdot 10^{-3}$ M; and Tris–HCl, 0.2 M (pH 7.5).

Hydroxylation system. 3–5 ml of substrate solution, 3–5 ml of enzyme extract and 1 ml of the solution of co-factors were incubated at 37° for 1 h in a Dubnoff incubator under air, with shaking. At the end of the reaction 100% trichloroacetic acid was added to give a 5% concentration. The mixture was then heated to 90° for 30 min for precipitation of noncollagenous proteins. The precipitate was extracted once more with 5% trichloroacetic acid, 90°, 15 min. After removal of the precipitate the excess trichloroacetic acid was removed by dialysis against running water overnight. The collagen extract was dried in a boiling-water bath under a stream of air and then hydrolyzed in 6 M HCl in a sealed tube at 110° for 16 h. The hydrolysate was evaporated to dryness. The residue was dissolved in 200 μl of water. A 10-μl aliquot was transferred to a 1-inch^2 of Whatman No. 4 paper and this was used for the determination of total counts as described below. The remainder of the hydrolysate was used for paper chromatographic separation of proline and hydroxyproline using Whatman No. 4 paper and 75% phenol as a developing agent in a descending system. Proline and hydroxyproline standards were run concurrently on adjacent strips for aid in localizing the amino acids. After development, chromatograms were cut into 0.5-inch sections and counted in a Packard Model 2111 automatic liquid scintillation spectrometer. The scintillator solution consisted of 3 g 2,5-diphenyloxazole and 0.1 g 1,4-bis-[2-(4-methyl-5-phenyl-oxazolyl)]-benzene per l of toluene. Counts above background in each section

* All manipulations were carried out at 5°.

TABLE I

THE EFFECT OF ENZYMIC CONCENTRATION ON THE HYDROXYLATION OF PROTOCOLLAGEN

The incubation mixture consists of 3 ml of substrate (10 000 disint./min per ml), 5 ml of enzymic solution and 0.9 ml of co-factors, 37°.

*Enzymic protein** (*mg/incubation*)	*Disint./min*			*% Hydroxylation*
	Hydroxyproline	*Proline*	*Total*	
0	388	19 600	19 988	1.94
2.87	225	9 470	9 695	2.32
5.74	626	11 300	11 926	5.25
11.48	1350	11 300	12 650	10.67
22.96	3080	11 100	14 180	21.73
45.92	4140	9 590	13 730	30.15
91.84	4380	9 100	13 480	32.49
143.50	4650	8 850	13 500	34.44

* The protein was determined by the method of LOWRY *et al.*[12].

under the peak areas of hydroxyproline and proline, respectively, were added. The percent hydroxylation was calculated as disint./min in hydroxyproline × 100/total disint./min of hydroxyproline *plus* proline. The counting efficiency for ^{14}C was 80% and individual sample counts varied from 3 times to 500 times background.

RESULTS

In Table I are shown data demonstrating the presence of protocollagen hydroxylase from pig uterus and the effects of enzyme concentration on the percent of hydroxylation of protocollagen. There is a straight line relationship between the degree of hydroxylation and the concentration of enzyme protein up to 23 mg protein per incubation. The data also indicate that proteolytic activity was present in the crude uterine extract which resulted in lower recovery of total counts for experimental samples than those found in control specimens. This was probably due to digestion of the protocollagen substrate during the hydroxylase reaction.

TABLE II

EFFECT OF TEMPERATURE AND GAS ATMOSPHERE ON HYDROXYLATION OF PROTOCOLLAGEN

The incubation mixture consists of 5 ml of substrate (3000 disint./min per ml), 5 ml of enzymic extract (24 mg protein per ml) and 1.1 ml mixture of co-factor. Data are average of 2 determinations. Deviations from the mean are less than 10%.

Atmosphere	*Temp.*	*Disint./min*			*% Hydroxylation*
		Hydroxyproline	*Proline*	*Total*	
Air	5°	79	4600	4679	1.69
Air	37°	739	4130	4869	15.18
95% O_2–5% CO_2	37°	608	3740	4348	13.98
N_2	37°	206	3060	3266	6.31

The requirement for O_2 for the hydroxylation reaction in uterine tissue is shown in Table II. A low level of hydroxylation (1.68%) was observed with the system at 5° under air. At 37° under air the degree of hydroxylation was 15%. The hydroxylation was the same under 95% O_2 and 5% CO_2 as under air. Under N_2 atmosphere the percent of hydroxylation was reduced to less than one-half that of the control, under air, at 37°.

TABLE III

CO-FACTOR REQUIREMENT OF PROTOCOLLAGEN HYDROXYLASE OF PORCINE UTERINE EXTRACT

The incubation mixture consists of 5 ml substrate (10 000 disint./min per ml), 3 ml of enzymic extract (23 mg protein per ml) and 1 ml of mixture of co-factor. Data are average of 2 determinations. Deviations from the mean are less than 10%.

Co-factors	*Disint./min*			*% Hydroxylation*
	Hydroxyproline	*Proline*	*Total*	
Complete*	11 500	20 600	32 100	35.82
— ascorbic acid	3 110	23 400	26 510	11.73
— $FeSO_4$	201	21 200	21 401	0.94
— α-ketoglutarate	2 600	22 500	25 100	10.36
— ascorbic acid, $FeSO_4$ and α-ketoglutarate	66	22 400	22 466	0.29

* See text for the complete composition of co-factors.

The co-factor requirements for the hydroxylation reaction is shown in Table III. Hydroxylation in the complete system was approx. 36%. Elimination of either ascorbic acid or α-ketoglutarate reduced the hydroxylation to 1/3 of that in the complete system. Omission of $FeSO_4$ decreased hydroxylation to 1/40 of that with the complete system. With the elimination of all three co-factors, the hydroxylation was less than 1% of that of the complete system. The differences in the requirement of each co-factor is probably due to the presence of a limited amount of these co-factors in the crude enzymic extract.

DISCUSSION

Data here reported indicate the presence of a highly active protocollagen hydroxylase in pig uterine homogenate. Up to 36% hydroxylation was obtained with the crude enzymatic extract of pig uterus compared to the theoretically maximal hydroxylation of about 43%. KIVIRIKKO AND PROCKOP[13] reported 15–23% hydroxylation in crude preparations of protocollagen and suggested that the observed hydroxylation corresponded to complete hydroxylation of the appropriate proline residues in the protocollagen. The higher levels of hydroxylation observed in the present experiments is probably due to partial purification of the hydroxylated protocollagen in the hot trichloroacetic acid step. The uterine tissue could be stored in the deep freeze for several weeks without a decrease in enzyme activity. The hydroxylase present in the pig uterine homogenate appears to be comparable to that of the chick embryo, in that atmospheric O_2 is required and that Fe^{2+}, ascorbic acid and α-ketoglutarate are co-

factors required for maximum activity. Whether there is an absolute requirement for these co-factors, and perhaps others not yet identified, must await purification of the enzyme. In experiments not reported here, we have observed that the uterine hydroxylase catalyzes the hydroxylation of both proline and lysine and can utilize as substrate a protocollagen extract from uterine tissue. Thus, the hydroxylating system of uterine tissue appears to be the same as that described in chick embryos, fetal skin and adult liver of rats and granulomas of guinea pigs. In preliminary experiments, there is some indication that the level of hydroxylase activity in the uterus is related to the stage of the estrus cycle. This is being further investigated.

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