ELEVATION OF PROLINE HYDROXYLASE ACTIVITY IN DISEASED RABBIT AORTA*

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Summary

Proline hydroxylase activity was demonstrated in 15,000 x g supernate of homogenates of rabbit aorta. The characteristics of the rabbit aorta enzyme were compared to those of rat liver, a known source of proline hydroxylase activity. Ascorbate, Fe^{+2} and α-ketoglutarate were found to be necessary for maximum activity. Kinetic data indicate that the apparent V_{max} and apparent Km for rabbit aorta enzyme and rat liver are similar in magnitude. These data confirm the presence of the collagen synthetic pathway in rabbit aortic tissue. Enzyme assays conducted on thoracic aorta indicated that proline hydroxylase activity is elevated six-fold in the presence of gross aortic plaquing.

Introduction

Collagen proline hydroxylase catalyzes the hydroxylation of specific peptidyl prolines in polypeptide precursors of collagen. This enzyme has been demonstrated in cell free homogenates of chick embryo (1), and in several vertebrate and invertebrate tissues (2,3,4). Enzyme from each of these reported tissue sources require atmospheric oxygen, α-ketoglutarate, iron and ascorbate for maximal hydroxylation of peptidyl proline.

Available evidence suggests that proline hydroxylase is a rate-limiting step in collagen synthesis (5,6). In our laboratory we are interested in studying the rate of collagen synthesis in rabbit aortae which have been injured by the simultaneous administration of epinephrine and L-thyroxine.

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Lorenzen (7) has reported that epinephrine-thyroxine injured aorta develop a plaque which is similar to the plaques found in human arteriosclerotic arteries. As part of long-range studies we have investigated the occurrence and characteristics of collagen proline hydroxylase in homogenates of rabbit aortae, from normal and treated rabbits.

Methods

Gross aortic plaques were induced in male New Zealand rabbits by the daily injection of epinephrine and thyroxine as described by Lorenzen (7).

Preparation of Enzyme Extracts. Aortae cleaned of adhering tissue from 10 male New Zealand rabbits weighing 2.5 to 3 Kg. were homogenized in 3 volumes of 0.25 M sucrose in a co-axial tissue grinder. The homogenate was centrifuged at 15,000 x g for 15 min. The pooled supernantant fluid contained 3-4 mg protein/ml and was used as the enzyme source. Livers from male, 120 gram Sprague-Dawley derived rats were homogenized in 2 volumes of 0.25 M sucrose, and centrifuged at 15,000 x g for 15 min. The rat liver supernant contained 20-30 mg protein/ml and was used as a reference enzyme source. Hutton and Udenfriend (2) have previously reported on the characteristics of proline hydroxylase obtained from adult rat liver.

Preparation of Labeled Substrate. Tritum labeled substrate was prepared using the method of Hutton et al. (8). 5-6 Grams of minced 10-11 day old chick embryos were incubated in 8.5 ml of modified Krebs-Ringer buffer in the presence of 500 μC of 3,4-^{3}H-l-Proline (New England Nuclear), and 1 mM α-α'-dipyridyl. The substrate was extracted in cold 0.5 N acetic acid and dialyzed against 3 changes of distilled water at 4°C. Substrate

prepared in this manner contained approximately 4 mg protein/ml with a specific activity of 100,000-150,000 dpm/mg protein. Protein was estimated by the method of Lowry et al. (9).

Hydroxylation System. Proline hydroxylase activity was assayed by the method of Hutton et al. (8). This assay system depends upon the formation of tritiated water (^{3}HHO) when peptidyl 3-4-^{3}H-proline is converted to peptidyl 3-^{3}H-hydroxyproline. Complete incubation mixtures contained 0.5 ml of labeled substrate, approximately 350,000 dpm, 7.5 µm ascorbic acid, 0.9 µm α-ketoglutarate, 0.45 µm ferrous ammonium sulfate, 0.5 M tris-HCl buffer pH 7.5, enzyme, and water to a final volume of 3 ml. Assay mixtures were incubated for 30 min. at 30°C and the reaction was stopped by adding 0.3 ml of 50% trichloroacetic acid (TCA). Tritiated water was assayed, after collection by vacuum distillation, in a Packard model 3310 liquid scintillation spectrometer. All data were corrected for background and are reported as DPM of ^{3}HHO, calculated by use of external standard.

Assay of Radioactive Hydroxyproline. ^{3}H-Hydroxyproline formed in the incubation of the above substrate with enzyme was measured by chilling the incubation mixtures and precipitating the protein with 0.3 ml of 50% trichloroacetic acid. The mixtures were centrifuged and the supernant was collected and assayed for ^{3}HHO content. The pellets were washed once with 10 ml of cold 5% trichloroacetic acid and the collagen was extracted into hot trichloroacetic acid (10). ^{3}H-Hydroxyproline was isolated as the free amino acid by column chromatography of protein hydrolyzates on columns of Dowex 50W-X8, 200-400 mesh eluded with 1N HCl (11). These columns were previously calibrated using ^{14}C-proline and ^{3}H-hydroxyproline standards. The first 40 ml fraction obtained from the columns contained

essentially all the hydroxyproline with proline appearing in the 60-80 ml fraction. The hydroxyproline fraction was evaporated to dryness, redissolved in 2 ml of water, and 1 ml was counted in a liquid scintillation spectrometer. Recovery of hydroxyproline from the columns was estimated by assaying known samples of ^{3}H-hydroxyproline. Recovery of ^{3}H-hydroxyproline was found to be 83%.

Results and Discussion

Rat liver enzyme was used to establish the relationship of ^{3}HHO formation to ^{3}H-hydroxyproline formation in substrate prepared in our laboratory. When ^{3}HHO and hot trichloroacetic acid soluble peptide bound ^{3}H-hydroxyproline (isolated as the imino acid) were assayed in the same incubation mixture, we observed a dependent and stoichiometric relationship as previously described (8). ^{3}HHO water formation in samples with

TABLE I

EFFECT OF ALTERED CO-FACTOR LEVELS ON THE PRODUCTION OF ^{3}HHO BY RAT LIVER PROLINE HYDROXYLASE

Alterations in Complete System	DPM ^{3}HHO / Mg. Enzyme	Percent of Control
None	1497	100
Ascorbate (3.75 μm/3 ml)	1721	115
Ascorbate (11.25 μm/3 ml)	1173	78
α Ketoglutarate (0.45 μm/3 ml)	1331	89
α Ketoglutarate (1.35 μm/3 ml)	1585	105
Ferrous ion (0.07 μm/3 ml)	1897	127
Ferrous ion (0.87 μm/3 ml)	1573	105

All samples contained 0.87 mg protein of the rat liver enzyme preparation; a complete system is defined in the text.

altered co-factor levels indicates that all co-factors of the "complete system" were present in excess amounts (Table I). In Table II the co-factor requirements of rat liver and rabbit aorta proline hydroxylase are compared. Omission of any of the co-factors resulted in a 60-90% decrease of enzyme activity. Similarly, omission of enzyme or replacing enzyme with boiled enzyme resulted in more than an 80% decrease in ^{3}HHO formation by both enzyme sources.

TABLE II

REQUIREMENTS FOR FORMATION OF TRITIATED WATER FROM PEPTIDYL 3-4-^{3}H-PROLINE USING RAT LIVER OR RABBIT AORTA PROLINE HYDROXYLASE

Omission from Complete System	Rat Liver		Rabbit Aorta	
	DPM ^{3}HHO	Percent of Control	DPM ^{3}HHO	Percent of Control
None	2936	100	2988	100
Ascorbate	352	12	533	17
Ferrous ion	1165	40	946	32
α Ketoglutarate	448	15	557	19
Ascorbate, Ferrous ion, and α Ketoglutarate	446	15	437	15
Enzyme	332	11	466	16
Enzyme replaced with boiled enzyme	450	15	428	14

The amount of enzyme preparation present was: rat liver 1.15 mg protein and rabbit aorta 0.76 mg protein. A complete system is defined in the text.

Series of samples containing varying amounts of substrate were incubated in the presence of a constant amount of enzyme preparation. The reciprocal of ^{3}HHO activity was taken as 1/V and plotted against the

reciprocal of ml of substrate as 1/S. Double reciprocal plots of both rabbit aorta and rat liver proline hydroxylase were found to be linear. The calculated apparent Km and V_{max} of each enzyme were similar in magnitude (Figure I).

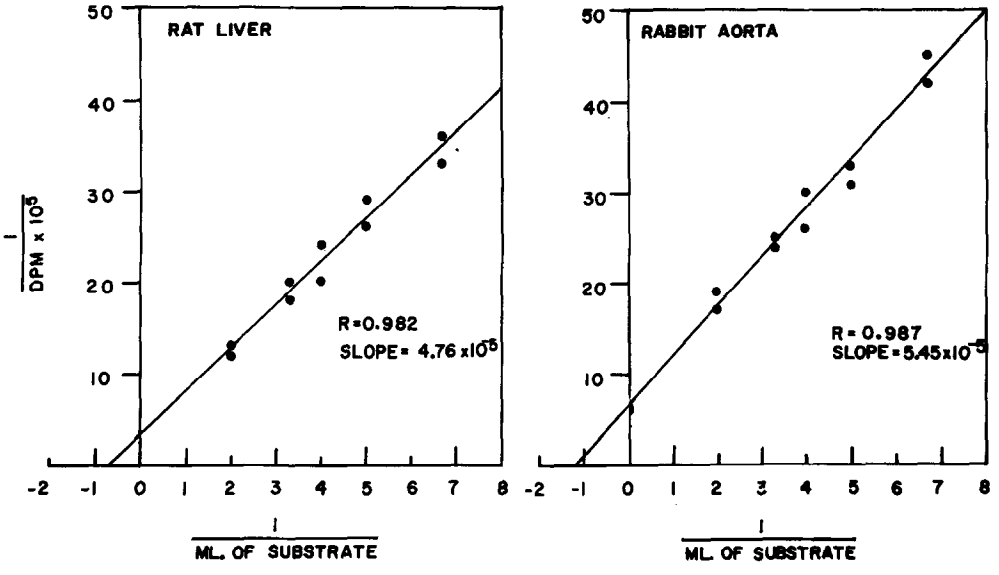

Figure I Effect of substrate concentration on reaction velocity of rat liver and rabbit aorta proline hydroxylase. The amount of enzyme preparation present was: rat liver 5.77 mg protein, and rabbit aorta 1.9 mg protein. The apparent Km for rabbit aorta was $\frac{0.438 \text{ ml substrate}}{\text{mg protein}}$ and for rat liver $\frac{0.23 \text{ ml substrate}}{\text{mg protein}}$ The apparent V_{max} for rabbit aorta was $\frac{8,096 \text{ DPM}}{\text{mg protein}}$ and for rat liver $\frac{5,613 \text{ DPM}}{\text{mg protein}}$.

The amount of tritiated water formed in the presence of varying amounts of enzyme was assayed. ^{3}HHO formation was linear in relation to enzyme concentration over a limited range (Figure II). The deviation at higher enzyme concentrations occurs as the substrate becomes limiting. In both cases the deviation from linearity occurs at approximately 5,000 dpm of ^{3}HHO. As shown in Figure II. the specific activity of rabbit aortae

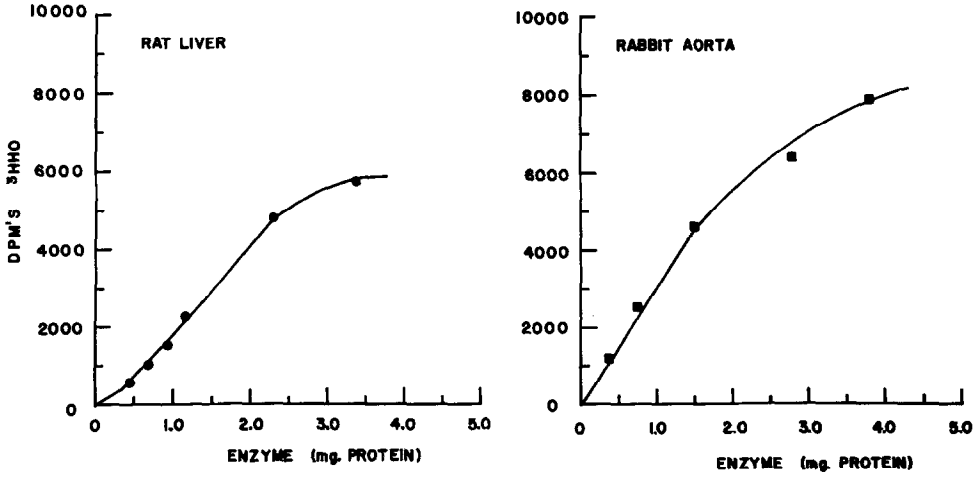

Figure II Effect of rabbit aorta and rat liver proline hydroxylase concentration on the formation of ^{3}HHO from peptidyl 3-4-^{3}H-proline substrate.

enzyme is slightly greater than rat liver proline hydroxylase.

These data confirm the presence of the collagen synthetic pathway in rabbit aortic tissue. The proline hydroxylase of rabbit aortic tissue appears to be comparable to that reported in other tissues in that iron, ascorbic

TABLE III

PROLINE HYDROXYLASE ACTIVITY OF THE THORACIC AORTA FROM CONTROL AND EPINEPHRINE- THYROXINE TREATED RABBITS

Treated DPM ^{3}HHO / mg protein	Controls DPM ^{3}HHO / mg protein
Mean Values $\pm$ S.E.	
*(6) 14,887 $\pm$ 1022	*(8) 2508 $\pm$ 368

*Number of animals assayed

The portion of the rabbit aorta from the aortic arch to the level of the coeliac artery was used as the thoracic aorta.

acid and α-ketoglutarate are necessary co-factors for maximal activity.

The above assay system was employed to measure proline hydroxylase activity in the thoracic aorta of control and diseased rabbits. Rabbits treated for 15 days with toxic doses of epinephrine (I. V.) and L-thyroxine (I. P.) developed gross aortic plaques and exhibited a six-fold increase in collagen proline hydroxylase activity (Table III). Further studies are currently being conducted to explore the relationship of the observed increase in proline hydroxylase to aortic plaque development.

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