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DIFFERENT RESPONSES TO SOME STIMULATORS OF PROLYL HYDROXYLASE ACTIVITIES IN VARIOUS RAT ORGANS

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Summary

Prolyl hydroxylase (proline,2-oxoglutarate dioxygenase, EC 1.14.11.2) of soluble fraction (105 000 × *g* supernatant) of rat granulation tissues was markedly enhanced by addition of nucleoside triphosphates to the assay medium. But the stimulatory activities of nucleoside triphosphates were very different in fractions derived from tissues of rat. In skin, lung or whole fetal tissues other than granuloma, GTP enhanced the enzymatic activity by 3—4 fold. On the other hand, in kidney, liver and spleen tissues it brought about no enhancement. The same results were obtained even if ATP regenerating system was added in the assay medium. The stimulatory effect of nucleoside triphosphates was not seen with the soluble fraction of liver, but it appeared with the enzyme fraction purified by affinity column chromatography. The same phenomenon was observed by addition of bovine serum albumin instead of nucleoside triphosphates as stimulator. We discuss the possible reasons as to why the responses of the enzyme to stimulators were quite different among various tissues.

Introduction

The biosynthesis of collagen involves several unique enzymatic steps. One of these is catalyzed by prolyl hydroxylase (proline,2-oxoglutarate dioxygenase, EC 1.14.11.2), which converts certain proline residues, already in peptide linkage, to hydroxyproline residues [1—3]. The prolyl hydroxylase, one of the new and unusual classes of mixed function oxygenases, requires α-ketoglutarate [4], ferrous ion [5,6], ascorbate [5,6], and atmospheric oxygen [8]. As purification of the enzyme progressed, some additional factors such as serum albumin, dithiothreitol [9,10] and catalase [12], were found to stimulate

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enzymatic activities even in the presence of cofactors. How these substances stimulate enzymatic activities is not yet clear.

In addition to the various requirements for hydroxylase activity already listed, we have found that the activity of prolyl hydroxylase purified from chick embryo is greatly stimulated by the presence of nucleoside triphosphates in the assay mixture [13]. Among nucleoside triphosphates, GTP was the most effective followed by ATP and ITP, while the pyrimidine nucleotides were less effective. Nucleoside di- and monophosphates showed no effect on the stimulation. dATP and ATP analogues such as adenosine 5'-(β,γ-imino) triphosphate, adenosine 5'-(β,γ-methylene) triphosphate, etc. were inactive.

In the present study, we further found that the stimulatory activities of nucleoside triphosphates were very different in fractions derived from tissues of rat if the protocollagen was hydroxylated by using soluble fractions (105 000 $\times g$ supernatant) as an enzymatic source instead of the purified enzyme. A possible reason of these different responses of prolyl hydroxylase in soluble fractions of various tissues of rat was discussed.

Materials and Methods

L-[4-^{3}H]Proline (21 Ci/mmol) was a product of Shwarz. ATP, GTP and poly-(L-proline) were obtained from Sigma Chemical Company. Bovine serum albumin was purchased from Miles Laboratories. Dithiotreitol, phosphoenol pyruvate kinase were obtained from Boehringer, Mannheim.

Assay of enzyme. Prolyl hydroxylase activity was assayed essentially according to the method of Hutton, et al. [14]. Each assay mixture had a volume of 1 ml and contained the following: Tris · HCl buffer, pH 7.5, 200 μmol; $FeSO_4$, 0.2 μmol; sodium ascorbate, 10 μmol; α-ketoglutarate, 2 μmol; catalase, 200 μg; peptidyl proline [4-^{3}H]labelled substrate (60 000 cpm); enzyme preparation (about 100 μg of protein). The substrate was prepared with minced rat embryos according to the same authors, in which L-[4-^{3}H]proline had been incorporated as protocollagen-proline under the presence of α,α'-dipyridyl to prevent hydroxylation. The reaction was carried out for 15 min at 37° C aerobically with shaking and stopped by adding 0.1 ml of 50% trichloroacetic acid. Tritiated water formed was collected by vacuum distillation. Radioactivity was determined in a liquid scintillation spectrometer using Bray's solution. Blanks were within 30 cpm.

Preparation of soluble fractions from various tissues of rats. All procedures were performed at 0—4° unless otherwise noted. Various tissues (brain, heart, kidney, lung, spleen, muscle, skin and thymus) were removed from 6-week Donryu rats (4 rats), and washed twice with a solution containing 0.25 M sucrose, 50 mM Tris · HCl, pH 7.5, and 0.02 mM KCl. Tissues were minced, resuspended in 3 vols sucrose buffer and homogenized in a Vir Tis "45" homogenizer at maximum speed for 45 s and homogenized again in a motor-driven Teflon pestle-glass homogenizer (5 strokes). The homogenates were centrifuged at 15 000 $\times g$ for 10 min to remove intact cells, nuclei, and mitochondria. The supernatants from this step were centrifuged at 105 000 $\times g$ for 1 h to obtain the S-105 fractions.

Preparation of purified enzyme fractions from rat liver and granuloma.

Prolyl hydroxylase was purified by affinity chromatography according to the modified method of Tuderman et al. [15]. All procedures were carried out at 0—4°C. An aliquot of the S-105 fractions of rat liver or granuloma were precipitated by adding solid ammonium sulfate to a saturation of 30% (17.6 g/100 ml). The pellet was removed by centrifugation at 15 000 × *g* for 30 min, and the supernate was precipitated with solid ammonium sulfate at a concentration of 65% saturation (23.5 g/100 ml). The pellet was dissolved in a solution consisting of 0.1 M NaCl, 0.1 M glycine and 0.01 M Tris · HCl buffer, pH adjusted to 7.5. The sample was dialyzed for 4 h against two changes of this solution. The sample was centrifuged again to remove a small amount of insoluble material, and then adjusted to a protein concentration of 10 mg per ml with buffer. An aliquot of the diluted enzyme fraction was passed through an affinity column containing poly(L-proline), molecular weight 30 000, linked to agarose (BrCN activated), having a bed volume of 5 ml. The column was equilibrated with a solution comprising 0.1 M glycine, 10 μM dithiothreitol and 0.01 M Tris · HCl buffer adjusted to pH 7.5 at 4°C and the sample was passed through the column at a flow rate of about 10—20 ml/h. After the sample had passed through the column, the column was washed with the equilibrating solution until the absorbance at 280 nm was about 0.01. The enzyme was then eluted with 0.01 M sodium acetate buffer (pH 4.0) containing 0.2 M NaCl. The fractions, collected in tubes, were immediately neutrality neutralized with NaOH. The fractions containing most of the enzymatic activity were pooled and stored at −20°C. The specific activity was 2000-fold greater than that of the original 105 000 × *g* supernatant fraction.

Preparation of carrageenin granuloma of rat. Granuloma pouch was induced by the modification of Selye's method using carrageenin as a phlogistic agent [16,17]. Animals used were male rats of Donryu strain 42 ± 3 days old. A 2% (w/v) solution of carrageenin (TS-36, kindly supplied by Taisho Pharmaceutical Co., Ltd., Tokyo) in 0.9% NaCl was sterilized by heating at 120°C for 30 min. Antibiotics were added before injecting the solution (0.1 mg penicillin G potassium and 0.1 mg dihydrostreptomycin sulfate per ml of the solution). 6 ml air were injected subcutuaneously on the dorsa of animals 1 day before the injection of the carrageenin solution. Then 4 ml of the solution were injected into the air sac already formed. On day 6 the animals were killed and the entire fluid in the granuloma pouch was harvested, then the capsule of the granulomatous tissue was carefully removed.

Results and Discussion

Additon of ATP in the enzyme assay system resulted an enhancement of prolyl hydroxylase activity in the soluble fraction (S-105) of rat carrageenin granuloma by 2—3-fold. Similarly addition of GTP, which was more effective than ATP, enhanced it by 2.5—3.5-fold (Fig. 1A). The stimulatory effect of ATP or GTP was observed without a measurable lag when ATP or GTP was added to complete reaction mixture during the course of reaction. This stimulation effect was seen even at a nucleotide concentration as low as 0.1 mM, and maximum stimulation was attained at about 3 mM nucleotides (Fig. 1B). With addition of 3 mM GTP to the enzyme assay system of soluble fractions (S-105)

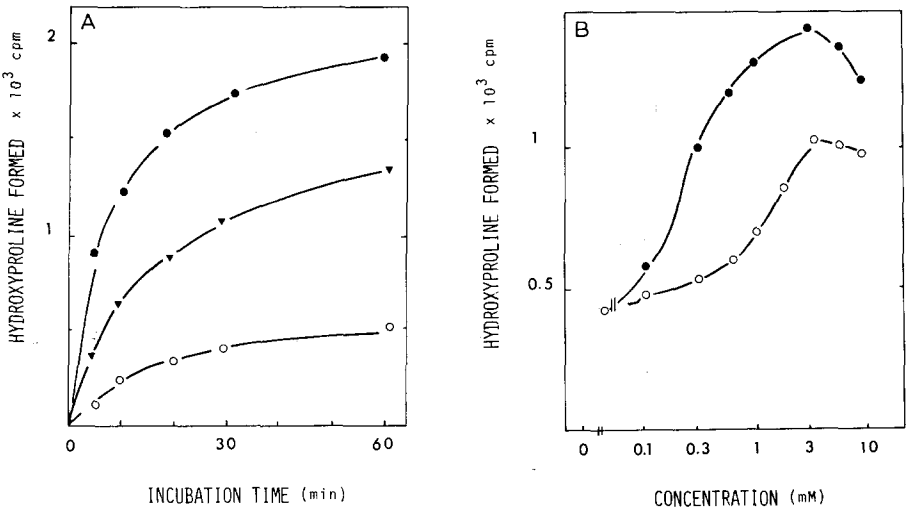

Fig. 1A. Time course of substrate hydroxylation by soluble fraction of rat granuloma in the presence of ATP or GTP. Enzyme activity was assayed with the standard assay system described in Materials and Methods. (○), none; (▼), ATP 3 mM; (●), GTP 3 mM. B. Effect of ATP or GTP concentration on the hydroxylation of protocollagen by soluble fraction of rat granuloma. (○), ATP; (●), GTP.

prepared from various tissues of rats, different responses to GTP were observed (Table I). Addition of GTP to S-105 fractions from whole fetal body, granuloma, skin and lung tissues enhanced the enzymatic activity by 3—4 fold. On the other hand, in the case of kidney, liver and spleen tissues it brought

TABLE I

DIFFERENT RESPONSES TO GTP OF NON-PURIFIED PROLYL HYDROXYLASE FROM VARIOUS TISSUES OF RATS

Soluble fraction of fetus, weighing about 1.5 g, was prepared as described in Materials and Methods. Soluble fraction of granuloma was prepared from capsule of the carrageenin induced granulomatous tissues as given in Materials and Methods. Lithium-salt GTP solution, neutralized with NaOH, was added in the assay medium to give a final concentration of 3 mM. 100—200 μl of solubel fractions from various tissues containing 100—300 μg protein was added in the assay medium. The values are averages of duplicates.

Experiment I			Experiment II	
Tissue	Specific activity (cpm/mg protein)	Stimulation activity (GTP/None (%))	Specific activity (cpm/mg protein)	Stimulation activity (GTP/None (%))
Brain	45	273	202	188
Heart	3324	225	1806	323
Kidney	360	122	362	67
Lung	5414	320	4310	240
Liver	1722	140	2882	84
Spleen	2840	121	2072	97
Muscle	1605	243	1210	161
Skin	3867	386	2000	392
Thymus	368	223	691	167
Granuloma	13552	384	8478	361
Fetus	12133	453	6933	357

about no enhancement of the enzymatic activity. The GTP concentrations giving maximal stimulation were all about 3 mM in various organs. Similar results were observed by using ATP instead of GTP. The effect of nucleoside triphosphates on the kinetics in regard to the co-substrates was the increase of *V*. We compared the enzymatic activities of soluble fractions of liver and granuloma to investigate why the responses of the enzyme to stimulators were quite different among various tissues.

Fluctuation of ATP concentration during incubation was investigated to clarify the possibility that ATP or GTP might be strongly broken down in the liver fraction. No difference was observed in the degradation activity of ATP between liver and granuloma. To confirm this point further, the stimulatory effect of ATP was examined with maintaining the ATP level at more than 80% during incubation by adding ATP regenerating system, i.e., phosphoenolpyruvate 1 mM, pyruvate kinase 40 μg/ml in the assay medium. Different responses were also observed between liver and granuloma even if ATP regenerating system was added in the assay medium. The enzymatic activity of the soluble fraction of liver attained 99% of the controls in the presence of ATP, and 88% in the presence of ATP regenerating system. On the other hand, in the case of granuloma it attained 263% and 257% of the controls, respectively. Consequently, this different responsiveness may depend on factors other than the degradation activity. We then purified the enzyme to investigate other factors which regulate the stimulating activity of nucleotides. More than 2000-fold purified enzyme fractions were obtained from soluble fractions of rat liver and granuloma by the affinity chromatography using poly(L-proline) as a ligand as described in details in Materials and Methods. As shown in Table II, addition of GTP to the enzyme fraction purified from liver soluble fraction resulted in about 3-fold enhancement of enzymatic activity. On the other hand, in the case of granuloma, GTP caused enhancement of enzymatic activity in both purified or non-purified fractons.

Bovine serum albumin, another stimulator of prolyl hydroxylase, gave results similar to those obtained with nucleotides. These results may suggest that: (1) there are substance(s) wich inhibit the stimulation effect of nucleotides or bovine albumin in the soluble fraction of liver; (2) there are substances which stimulate the enzymatic activity in the soluble fraction of liver in a manner

TABLE II

EFFECT OF GTP AND BOVINE SERUM ALBUMIN ON PURIFIED OR NON-PURIFIED PROLYL HYDROXYLASE FROM LIVER AND GRANULOMA OF RATS

Purified fraction was obtained by affinity chromatography as described in Materials and Methods. Values are means ± S.E. of three determinations.

Tissue	Enzyme fraction	3 mM GTP (GTP/None (%))	2 mg BSA (BSA/None (%))
Liver	S-105 fraction	122 ± 0.3	81 ± 1.0
	purified fraction	295 ± 5.2	219 ± 3.2
Granuloma	S-105 fraction	349 ± 0.3	224 ± 2.0
	purified fraction	293 ± 0.9	205 ± 1.2

Abbreviation: BSA, bovine serum albumin.

similar to nucleotides or bovine serum albumin. Addition of bovine serum albumin to the assay medium resulted in enhancement of the enzymatic activity proportional to the bovine serum albumin concentration, up to 6 mg/ml. In the presence of higher concentrations of bovine serum albumin, however, ATP was less active showing no further stimulation of the enzymatic activity [13]. Addition of liver soluble fraction, inactivated by heating at 50°C for purified enzyme by 2—3-fold. Soluble fractions of liver, kidney, and spleen, in which no enhancement of enzymatic activity occurred, may contain many stimulators such as albumin or albumin-like proteins. Similarly glutathione, reduced form, at 6 mM concentration enhanced the enzymatic activity markedly, and in this case too, ATP did not stimulate any further. Bovine serum albumin and glutathione, reduced form, can replace nucleoside triphosphates in enhancement of prolyl hydroxylase activity, which suggests that the effect is not a very specific one. Different responses in soluble fractions from various rat organs are probably to be explained more by differences in some effectors present than by heterogeneity of active prolyl hydroxylase protein. These observations may be taken to indicate that there are lots of substances which stimulate the enzymatic activity in the soluble fraction of liver, such as serum albumin, glutathione, cysteine and other SH-reducing substances. It is likely that in such tissues there is no further stimulation of prolyl hydroxylase by the stimulators.

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