

STIMULATION OF PROLYL HYDROXYLASE ACTIVITY BY BLEOMYCIN

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The activity of purified prolyl hydroxylase (proline, 2-oxoglutarate dioxygenase, EC 1. 14. 11. 2) was enhanced 3~8-fold at a low concentration of ferrous ion (1×10^{-5} M) by addition of bleomycin, a glycopeptide antibiotic with antineoplastic activity and a side effect of producing pulmonary fibrosis. The maximum stimulation was attained at a concentration of 15 $\mu\text{g}/\text{ml}$ bleomycin (about 1×10^{-5} M), which was approximately equimolar with the ferrous ion, one of the cofactors of this enzyme. Addition of bleomycin to the assay mixture resulted in a change of the optimal concentration of ferrous ion from 2×10^{-3} M to 1×10^{-5} M. Changing the order of addition of ferrous ion, enzyme and bleomycin in assay medium before incubation at 37°C, the stimulatory activity was varied. Bleomycin $\text{A}_2\text{-Cu}^{++}$ (Cu^{++} -chelated bleomycin), which scarcely complexed with Fe^{++} , had no enhancing effects on the enzymatic activity. We discuss the possible reasons as to why the activity of prolyl hydroxylase was enhanced by addition of bleomycin in the assay mixture.

Bleomycin, an antitumor antibiotic isolated from *Streptomyces verticillus* by UMEZAWA *et al.*,¹⁾ has been found to be a very useful drug against a number of human cancers, particularly human squamous cell carcinoma.^{2,3)} With increasing use, however, it has become apparent that diffuse pulmonary fibrosis is a severe and puzzling possible complication of bleomycin therapy.^{4,5)} The pathologic end stage of the toxic reaction, pulmonary fibrosis, has been described in both humans^{5,6)} and animals,⁷⁾ but few studies have been reported on the mechanism of fibrosis by bleomycin. TETSUKA *et al.* have reported that the drug enhanced collagen synthesis in tissue,⁸⁾ ICHIHASHI *et al.* have demonstrated the reduction of cutaneous collagenase activity after administration of the antibiotic,⁹⁾ and OTSUKA *et al.* have described the stimulation of the synthesis of acidic glycosaminoglycans in cultured fibroblasts.¹⁰⁾

In view of this, the present study was undertaken to attempt an analysis of bleomycin action. In this communication we report the effect of bleomycin on the purified prolyl hydroxylase (proline, 2-oxoglutarate dioxygenase, EC 1. 14. 11. 2.), which catalyzes the hydroxylation of peptidyl proline, one of the rate limiting steps in collagen biosynthesis.

Materials and Methods

Bleomycins were supplied by Nippon Kayaku Co. Ltd., Tokyo. [$4\text{-}^3\text{H}$] L-Proline (21 Ci/mmol) was a product of Schwarz Bio Research, Inc. Poly(L-proline) and superoxide dismutase were purchased from Sigma Chemical Company.

Enzyme assay

Prolyl hydroxylase activity was assayed according to the method of HUTTON *et al.*¹¹⁾ Each assay mixture had a volume of 1 ml and contained the followings: Tris-HCl buffer (pH 7.5) 200 μmol ;

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sodium ascorbate 2 μmol ; FeSO_4 0.01 μmol ; α -ketoglutarate 0.4 μmol ; catalase 200 μg ; peptidyl proline [$4\text{-}^3\text{H}$]-substrate (80,000 cpm); enzyme preparation (0.01 μg of protein). The substrate was prepared with minced rat embryos according to the same authors, in which [$4\text{-}^3\text{H}$] L-proline had been incorporated as protocollagen-proline in the presence of α, α' -dipyridyl to prevent hydroxylation. The reaction was carried out for 15 minutes 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 less than 30 cpm.

Preparation of purified enzyme

Prolyl hydroxylase was purified by affinity chromatography according to the modified method of TUDERMAN *et al.*¹²⁾. Rat fetuses were minced and homogenized in 3 volumes of buffer solution containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5) and 0.02 mM KCl with a Vir Tis "45" homogenizer at maximum speed for 45 seconds and homogenized again in a motor-driven Teflon pestle-glass homogenizer (5 strokes). The $105,000\times g$ supernatant was fractionated with ammonium sulfate (30~65% saturation). 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 7.5). An aliquot of the diluted ammonium sulfate fraction, adjusted to a protein concentration of 10 mg/ml with buffer, was passed through an affinity column containing poly(L-proline), molecular weight about 30,000, linked to agarose (BrCN), having a bed volume of 5 ml. The enzyme was eluted with 0.01 M sodium acetate buffer (pH 4.0) containing 0.2 M sodium chloride. The fractions containing most of the enzymatic activity, neutralized with NaOH, were pooled and stored frozen. The specific activity was 2,000-fold greater than that of the original $105,000\times g$ supernatant fraction.

Results and Discussion

As shown in Fig. 1, the requirement for ferrous ion of prolyl hydroxylase was decreased to about $1/100\sim 1/200$ by addition of bleomycin (15 $\mu\text{g}/\text{ml}$), a glycopeptide antibiotic, in the assay medium, *i.e.*, the optimal concentration of ferrous ion was changed from 2×10^{-3} M to 1×10^{-5} M. At the relatively low concentration of ferrous ion the enzymatic activity was markedly enhanced by addition of bleomycin. At 1×10^{-5} M ferrous ion concentration, the enzymatic activity in the presence of bleomycin was 5-fold higher than that of control. LINEWEAVER-BURK plots of the data showed that the control and bleomycin-stimulated activities exhibited no difference in the affinity of enzyme for substrate, and that the primary action of bleomycin was to increase V_{max} .

As shown in Fig. 2, the maximum stimulation was attained at 15 $\mu\text{g}/\text{ml}$ of bleomycin (about $1\times$

Fig. 1. Effect of ferrous ion concentration on the purified prolyl hydroxylase in the absence or in the presence of 15 $\mu\text{g}/\text{ml}$ bleomycin.

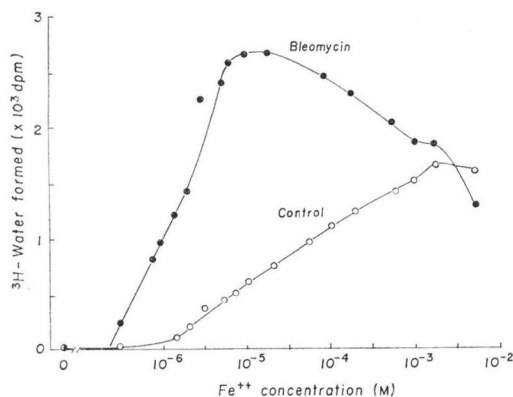


Fig. 2. Effect of bleomycin concentration on the purified prolyl hydroxylase.

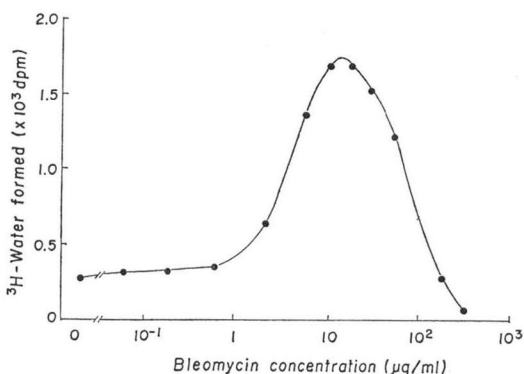
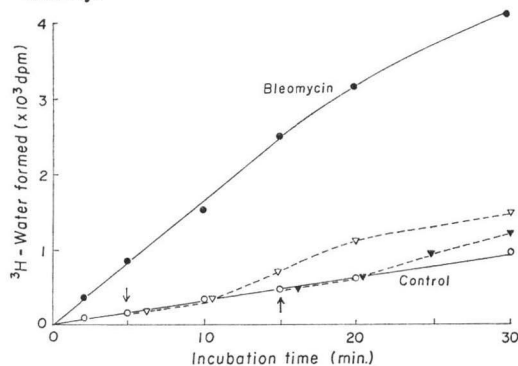


Fig. 3. Effect of addition of bleomycin during the course of incubation on the prolyl hydroxylase activity.



latory activity was decreased precipitously at concentrations greater than 15 $\mu\text{g/ml}$ of bleomycin. Higher concentrations of bleomycin were rather inhibitory to the enzymatic activity.

As shown in Fig. 3, there was a lag time in the appearance of stimulatory activity of bleomycin when it was added during the course of incubation, and the enzymatic activity was not so much enhanced during further 15-minute incubation with bleomycin. Changing the order of addition of ferrous ion, enzyme and bleomycin in assay medium before incubation at 37°C, the stimulatory activity was varied. For example, addition of bleomycin before enzyme caused 2-fold higher stimulation than the reverse. It may be important for bleomycin action that bleomycin interact first with cofactors, perhaps ferrous ion.

Optical spectral studies have revealed that a complex is formed between bleomycin and ferrous ion.¹³⁾

As shown in Table 1, addition of bleomycin A₂ resulted in an enhancement of prolyl hydroxylase activity by about 8-fold, but bleomycin A₂-Cu⁺⁺ (Cu⁺⁺-chelated bleomycin), a natural product isolated from *Streptomyces verticillus* had no effects on the enzymatic activity. Cupric ion alone, in amount equimolar with bleomycin A₂-Cu⁺⁺, did not interfere with the enzymatic activity. These results suggest that the ability of bleomycin to chelate ferrous ion may be important for the modification of the enzymatic activity.

From these observations it seems that bleomycin may interact with ferrous ion and that bleomycin-ferrous ion may affect the rate of hydroxylation of procollagen by prolyl hydroxylase. The stimulatory effect of bleomycin on prolyl hydroxylase was not inhibited by addition of superoxide dismutase, suggesting that neither O₂⁻ nor OH radical is the chemical mediator responsible for the stimulating action of bleomycin.

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Table 1. Effect of bleomycin A₂-Cu⁺⁺ on purified prolyl hydroxylase.

Addition*	Prolyl hydroxylase activity	
	dpm	% of control
None	606	100
Bleomycin A ₂	4,832	797
Bleomycin A ₂ -Cu ⁺⁺	571	94

* The concentration of bleomycins used was 15 $\mu\text{g/ml}$.

10⁻⁵), which was approximately equimolar with the ferrous ion in the assay medium. The stimu-

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