THE EFFECT OF BLEOMYCIN ON PROLYL HYDROXYLASE AND DNA CHAIN BREAKAGE: STRUCTURE-ACTIVITY RELATIONSHIP

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The activity of purified prolyl hydroxylase (proline, 2-oxoglutarate dioxygenase, EC 1. 14. 11. 2) was enhanced about 3-fold by addition of bleomycin in the assay mixture. Various members of the bleomycin family, their derivatives and degradation products were investigated for activities against prolyl hydroxylase together with their activities of DNA chain breakage to determine relationships between the structure of bleomycin and its various actions. All the bleomycins with various terminal amine parts and desamide bleomycin stimulated the enzymatic activity but did not exhibit an effect on DNA chain breakage. The stimulatory activity of bleomycin was not decreased by hydrolysis with 0.3 N H_2SO_4 at 80°C for 6 hours, conditions which liberates the sugar moiety, but was eliminated by hydrolysis with 6 N HCl at 105°C for 24 hours. In contrast both treatments decreased the DNA chain breakage activity of bleomycin. Optical spectral studies revealed that all the bleomycins and their hydrolysates which stimulated the prolyl hydroxylase activity made complexes with ferrous ion, one of the cofactors of this enzyme.

The bleomycins, isolated from *Streptomyces verticillus* by UMEZAWA *et al.*[1], are a family of glycopeptide antibiotics with antineoplastic activity. Bleomycin has been shown to have therapeutic value in the treatment of variety of neoplasms including squamous cell carcinomas[2,3]. With increasing use of bleomycin therapy diffuse pulmonary fibrosis has been recognized as a severe and puzzling complication.[4,5]

We have reported that the activity of purified prolyl hydroxylase [proline, 2-oxoglutarate dioxygenase, EC 1. 14. 11. 2], one of the modification enzymes of collagen biosynthesis, was enhanced about 3~5-fold by addition of bleomycin *in vitro*[6].

On the other hand, a major activity of the drug is the degradation of cellular DNA[7]. *In vitro*, DNA chain breakage is enhanced by reducing agents such as ascorbate, dithiothreitol, and 2-mercaptoethanol[8~11], by ferrous ion[12] and by creating an oxygen-saturated conditions[12,13]. We took notice of the similarities between the reaction of DNA chain breakage by bleomycin and the hydroxylation of peptidyl proline by prolyl hydroxylase, which requires α-ketoglutarate[14], ferrous ion[15,16], ascorbate[15,17], and the atmospheric oxygen[18].

In this communication, we report the actions of various bleomycin derivatives against prolyl hydroxylase together with their activities of DNA chain breakage and discuss the relationship between the structure of bleomycin and its actions.

Materials and Methods

Bleomycin A_2, other members of the bleomycin family and various derivatives were supplied by Nippon Kayaku Co., Ltd., Tokyo. These compounds were dissolved in water at a concentration of 150 μg/ml and the resulting solutions refrigerated until use. [4-^{3}H] L-Proline (21 Ci/mmol) was a

product of Schwarz Bio Research, Inc. [Methyl-^{3}H] thymine was obtained from the Radiochemical Centre Amersham, England. Poly (L-proline) was purchased from Sigma Chemical Company. *E. coli* C 600 T (−) was received from Dr. M. YAMADA, Department of Physiological Chemistry, Faculty of Pharmacology, University of Tokyo.

Preparation of labeled DNA:

Tritium-labeled DNA was prepared as described by MARMUR[19] from *E. coli* C 600 T (−), the DNA of which was labeled by incubation of the bacteria in a synthetic medium containing [methyl-^{3}H] thymine (1 μCi/ml).

Estimation of the rate of DNA chain breakage:

The activity of bleomycin was assayed by determining the proportion of labeled DNA soluble in 0.5 N trichloroacetic acid. The standard reaction mixture (0.5 ml), containing Tris-HCl buffer (pH 8.0) 50 μmol; dithiothreitol 0.03 μmol; bleomycin 0.1 μmol, and labeled *E. coli* DNA (5,000 dpm), was incubated at 30°C for 20 minutes. After the addition of 0.1 ml of carrier albumin and 0.1 ml of cold 35% trichloroacetic acid, the supernatant was counted in 10 ml of BRAY's solution.

Assay of enzyme:

Prolyl hydroxylase activity was assayed essentially according to the method of HUTTON *et al.*[20] Each assay mixture had a volume of 1 ml and contained the following: Tris-HCl buffer (pH 7.5) 200 μmol; sodium ascorbate 2 μmol; $FeSO_4$ 0.01 μmol; α-ketoglutarate 0.4 μmol; catalase 100 μg; peptidyl proline [4-^{3}H]-substrate (300,000 dpm); enzyme preparation (0.01 μg of protein). 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. Prolyl hydroxylase was purified from rat fetuses by affinity chromatography as described in the previous report[6].

Spectroscopic studies:

Spectroscopic measurements were performed on a Hitachi Recording Spectrophotometer 323. Thunberg cell with 10-mm light path was used.

Results and discussion

The effect of bleomycins with various terminal amine residues on prolyl hydroxylase was investigated and the results are shown in Table 1 together with their activities of DNA chain breakage. All the bleomycins tested with the different terminal amine substitution had stimulatory activities for prolyl hydroxylase. These results suggest that terminal amine parts are not important for this activity. On the other hand, the activities of bleomycins to break bacterial DNA chain were somewhat different, *e.g.*, demethyl A_2 and A_{5033} were 2 times weaker than bleomycin A_2.

As shown in Table 2, the stimulatory activity of bleomycin A_2 against the prolyl hydroxylase was decreased to 1/2 by prior hydrolysis with 6 N HCl at 37°C for 24 hours in a sealed tube. Hydrolysis with 6 N HCl at 105°C for 24 hours caused the loss of this activity. The hydrolysate, obtained by the latter procedure, contained at least 7 ninhydrin-positive products in agreement with the result of TAKITA, *et al.*[21]. The stimulatory activity of bleomycin was not decreased by hydrolysis with 0.3 N H_2SO_4 at 80°C for 6 hours, which liberates the sugar moiety, 2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-gulose[22]. In contrast all three treatments decreased the DNA chain breakage activity of bleomycin. These observations indicate that the sugar moiety of bleomycin molecule may play an important role in DNA chain breakage, but that it is not necessary for the stimulation of prolyl hydroxylase activity. The structure of the peptide part of bleomycin is indispensable for both activities.

As shown in Table 3, desamide bleomycin, lacking β-aminoalanine amide moiety of bleomycin,

has the same activity as bleomycin A_2 in stimulating the prolyl hydroxylase activity, but its activity in breaking DNA chain was 1/25 of bleomycin A_2 in agreement with the result of UMEZAWA, *et al.*[23]. Bleomycin A_2-Cu^{++} and bleomycinic acid-Cu^{++}, which are copper-chelated bleomycins, were inactive against both prolyl hydroxylase and DNA chain breakage. As the prolyl hydroxylase requires ferrous ion in addition to α-ketoglutarate, ascorbate and molecular oxygen as cofactors, it is possible that Cu^{++}-chelated bleomycins which forms complexes with ferrous ion with difficulty would be inactive against prolyl hydroxylase. Further, we found that addition of bleomycin in the assay mixture of the enzyme resulted in the decrease of the optical concentration of ferrous ion[6]. Therefore we investigated the binding activities of

Table 1. Effect of bleomycins with various terminal amine parts on prolyl hydroxylase and DNA chain breakage.

Prolyl hydroxylase activity and DNA chain breakage were assayed with standard assay conditions as described in Materials and Methods. Values are means±S.E. of three determinations. The concentration of bleomycins used was 15 μg/ml.

Bleomycins	Prolyl hydroxylase activity		DNA chain breakage	
	dpm	% of control	dpm	% of A_2
None (Control)	562±27	100	103± 18	2
A_2	1,718±40	306	4,716±141	100
Demethyl A_2	1,893± 9	337	1,874±744	40
A_5	1,781±45	317	4,452±157	94
B_4	1,576±45	280	4,495±867	95
TMD	1,620±40	288	3,091±442	66
A_{5033}	1,776±31	316	2,021±452	43

Table 2. Effect of bleomycin hydrolysate on prolyl hydroxylase and DNA chain breakage.

Prolyl hydroxylase activity and DNA chain breakage were assayed with standard assay conditions as described in Materials and Methods. After bleomycin A_2 was hydrolyzed in a sealed tube, hydrolysates were neutralized with NaOH. The concentration of bleomycin A_2 or its hydrolysates was 15 μg/ml.

Bleomycin A_2 treatment	Prolyl hydroxylase activity		DNA chain breakage	
	dpm	% of control	dpm	% of no treatment
None (Control)	603	100	111± 17	3
No treatment	2,018	335	3,684±273	100
6 N HCl, 37°C, 24 hours	1,134	188	175± 25	5
6 N HCl, 105°C, 24 hours	567	94	198± 51	5
0.3 N H_2SO_4, 80°C, 6 hours	1,844	306	236± 20	6

* Values are average of duplicate samples.
** Values are means±S. E. of 3 determinations.

Table 3. Effect of bleomycin derivatives on prolyl hydroxylase and DNA chain breakage.

Prolyl hydroxylase activity and DNA chain breakage were assayed with standard assay conditions as described in Materials and Methods except that Fe^{++} concentration in the enzyme assay mixture was 0.02 μmol/ml. The concentration of bleomycins used was 15 μg/ml. Values are means±S. E. of 3 determinations.

Bleomycins	Prolyl hydroxylase activity		DNA chain breakage	
	dpm	% of control	dpm	% of Blm A_2
None (Control)	606± 79	100	103± 18	2
Bleomycin A_2	4,832±163	797	4,716±141	100
Desamide-A_2	5,896± 86	972	199± 8	4
Bleomycin A_2-Cu^{++}	571± 23	94	170± 19	4
Bleomycinic acid-Cu^{++}	854± 53	140	215± 29	5

various bleomycins to ferrous ion by spectral studies according to the method of SAUSVILLE, *et al.*[12]. Optical spectral studies revealed that a complex was formed between bleomycin and ferrous ion. The complex, studied under both anaerobic and aerobic conditions in the presence of ascorbate, exhibited a broad maximum near 480 nm. All the bleomycins and their hydrolysates, which stimulated the prolyl hydroxylase activity, form complexes with ferrous ion. In other words the bleomycin derivatives and hydrolysates, which could not make complexes with ferrous ion, did not stimulate the enzymatic activity.

These results suggest that the elements of the bleomycin structure which will complex with ferrous ion are necessary for the stimulation of prolyl hydroxylase, and that neither β-aminoalanine amide moiety nor carbamoyl group in bleomycin play an important role in this action. The latter however are important for the effects on DNA chain breakage.

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