INHIBITION OF DOPAMINE-β-HYDROXYLASE, A COPPER ENZYME, BY BLEOMYCIN

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Bleomycin was found to be one of the most potent inhibitors of dopamine-β-hydroxylase. Bleomycin-A2 at 8×10^{-8} M inhibited the enzyme activity by 50%. Kinetic studies showed that the inhibition by bleomycin-A2 was of the competitive type with both the substrate and the cofactor, ascorbate, and was not affected by fumarate, a stimulator for the enzyme. The inhibition mechanism is possibly due to chelating action of bleomycin toward the copper atom at the active site of the enzyme together with some other kinds of binding, for the addition of the cupric ions or extensive dialysis completely reversed the inhibition and bleomycin Cu(II)-complex did not inhibit the enzyme.

Bleomycin (BLM), a water-soluble basic glycopeptide antibiotic, is isolated as the equimolar *Cu(II)*-complex from *Streptomyces verticillus*[1] and because of its potent antitumor activity, BLM has been widely used for the treatment of human squamous cell carcinoma. The chemical structure of BLM-copper complex was studied and determined by TAKITA *et al.*[2] It is known that BLM binds to DNA and causes DNA strands breakage, and Fe (II) and oxygen are required for the breakage of DNA. It has been proposed that superoxide and hydroxyradicals may play a role in this action[3]. BLM has a strong chelating activity with various metals, especially cupric ion. Copper-free BLM strongly binds with cupric ion and the equimolar complex is formed. The formation constant of the 1: 1 BLM Cu(II)-complex was determined to be $K=10^{12.63}$ [4]. Because of its strong chelating activity, BLM-radioactive metal complexes (BLM-^{57}Co, BLM-^{99m}Tc and BLM-^{111}In) are used in nuclear medicine to detect lung cancers and brain tumors[5].

Dopamine-β-hydroxylase [3,4-dihydroxyphenylethylamine, ascorbate: O_2 oxidoreductase (hydroxylating), EC 1.14.17.1.] is a copper-containing monooxygenase, which requires ascorbic acid as a cofactor[6] and catalyzes the final step in the biosynthesis of norepinephrine. Since the enzyme is a copper enzyme, the effect of BLM on the activity is of our interest. The present communication describes the inhibitory action of BLM on pure dopamine-β-hydroxylase *in vitro*.

Materials and Methods

Copper-free BLM-A2, BLM acid, deamide-BLM-A2, BLM-A5, BLM-B2, BLM-B4 and pepleomycin (BLM-PEP) were kindly supplied by Nippon Kayaku Co. Ltd. Beef adrenal glands were obtained fresh, packed in ice, from the slaughterhouse. Dopamine-β-hydroxylase was homogenously purified from bovine adrenal medulla by the method of FOLDES *et al.*[7] The enzymic activity was

measured by spectrometry according to the procedure of KATO *et al.*, using tyramine as the substrate[8]. The standard reaction mixture for the enzymic assay (final volume 1.0 ml) contained, an appropriate amount (1 μg) of the purified enzyme solution, 100 μl of 2 M sodium acetate buffer (pH 5.0), 25 μl of aqueous solution (2 mg/ml) of catalase (2,500 U, 50 μg/ml), 50 μl of 0.2 M ascorbic acid, 50 μl of 0.2 M sodium fumarate and 50 μl of 0.4 M tyramine. The reaction was started by adding substrate and continued for 30 minutes at 37°C in air. When the effect of preincubation was examined, this reaction mixture was preincubated for 10 minutes at 37°C, then reaction was started by adding substrate. The reaction was stopped by the addition of 0.2 ml of 3 M trichloroacetic acid. As a control, reaction mixture without tyramine was incubated at the same time and substrate was added after stopping the reaction. The octopamine formed from tyramine was isolated on a small column of Dowex 50-H^+, and was assayed on an aliquot of the column eluate by periodate oxidation to *p*-hydroxybenzaldehyde followed by the measurement of the absorbance at 333 nm.

Results

1. Inhibition of Dopamine-β-hydroxylase by Bleomycin

As shown in Table 1, BLM derivatives inhibited dopamine-β-hydroxylase at a concentration as low as 10^{-8} M under a copper-free condition. For example, BLM-A2, which is the major component of natural BLM, inhibited the enzyme activity at a concentration of 8×10^{-8} M by 50% (Table 1). There was no significant difference in the inhibitory action among these BLMs. Preincubation of the enzyme with BLM at various concentrations (1×10^{-9} M, 1×10^{-8} M, 3×10^{-8} M and 5×10^{-8} M) did not affect the extent of inhibition. Since dopamine-β-hydroxylase is a copper-containing enzyme, the possibility that BLM may inhibit the enzyme by the chelation with the copper of the enzyme was examined. As also shown in Table 2, the inhibition was completely reversed by the addition of copper. Addition of greater amounts of copper causes inhibition of the enzyme. Therefore, the effect of copper at concentrations higher than 1×10^{-6} M could not be studied in this experiment. Further examination was carried out by using BLM-A2, the major component of natural BLM.

Table 1. Inhibition of dopamine-β-hydroxylase by bleomycin.

	BLM concentration at 50% inhibition (M)
BLM acid	2.7×10^{-8}
BLM-A2	8.0×10^{-8}
BLM-A5	6.3×10^{-8}
BLM-B2	2.6×10^{-8}
BLM-B5	6.4×10^{-8}
BLM-PEP	3.5×10^{-8}
Deamide-BLM-A2	3.8×10^{-8}

BLM: Bleomycin, BLM-PEP : Pepleomycin

Table 2. Inhibition of dopamine-β-hydroxylase by BLM-A2.

BLM-A2 concentration (M)	Dopamine-β-hydroxylase activity (% of control activity)	
	Enzyme alone	Enzyme+Cu^{2+} 1×10^{-6} M
1×10^{-9}	99	100
1×10^{-8}	99	100
5×10^{-8}	92	100
1×10^{-7}	29	100
2×10^{-7}	10	100
5×10^{-7}	3	100
1×10^{-6}	1	98
2×10^{-6}	0	8
5×10^{-6}	0	2
1×10^{-5}	0	1

2. Mechanism of the Effect of BLM on Dopamine-β-hydroxylase

Reversal of BLM-A2 inhibition of dopamine-β-hydroxylase by dialysis was examined. The inhibition caused by BLM-A2 was completely reversed by dialysis against phosphate buffer, indicating the inhibition is reversible (Table 3).

Fig. 1. LINEWEAVER-BURK plots of ascorbate concentrations against rate of hydroxylation with and without BLM-A2.

Reaction mixture contained 0.2 M sodium acetate buffer (pH 5.0), catalase (50 μg/ml), 10 mM fumarate, 20 mM tyramine, an appropriate amount of enzyme and varying amounts of ascorbate.

BLM-A2 was added without preincubation. Incubation was continued at 37°C for 30 minutes. Assay was initiated by addition of tyramine and carried out as described in Materials and Methods.

The velocities(v) are expressed as μ moles of octopamine formed from tyramine for 30 minutes. ○—○, enzyme alone; □—□, enzyme with BLM-A2 3×10^{-8} M, ●—●, enzyme with BLM-A2 5×10^{-8} M.

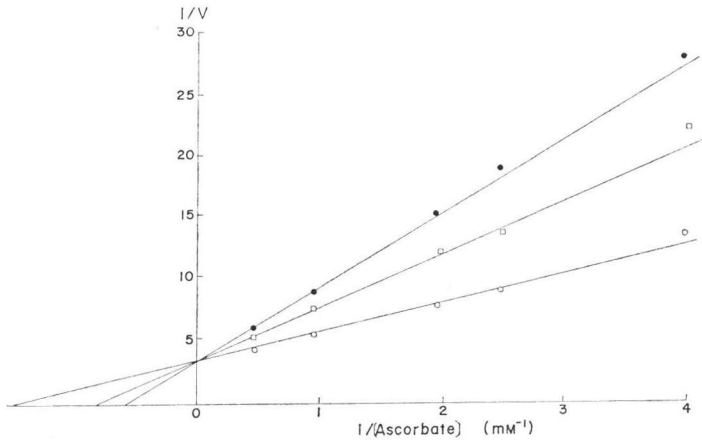

Table 3. Reversal of BLM-A2 inhibition of dopamine-β-hydroxylase by dialysis.

	Dopamine-β-hydroxylase (% of control activity)	
	Before dialysis	After dialysis
Control	100	100
BLM treated enzyme	29	95

Enzyme was preincubated with BLM-A2 (1×10^{-7} M) for 10 min. at 37°C in the presence of catalase (50 μg/ml). 800 μl of the aliquot was used for the assay. Dialysis was carried out against 2,000 volumes of 10 mM phosphate buffer (pH 7.2) at 4°C for 2 hours, buffer was changed at 1 hour.

Table 4. Effect of fumarate concentrations on the inhibition by BLM-A2 of dopamine-β-hydroxylase activity.

Fumarate concentration (M)	Dopamine-β-hydroxylase activity (% of control activity)		
	Enzyme alone	Enzyme+BLM-A2 (M)	
		5×10^{-8}	8×10^{-8}
1×10^{-2}	100	45	28
1×10^{-3}	83	35	25
5×10^{-4}	64	25	19
0	57	19	13

The kinetics of BLM-A2 inhibition on dopamine-β-hydroxylase were studied by using LINEWEAVER-BURK plots to determine the type of inhibition. As shown in Fig. 1, inhibition of dopamine-β-hydroxylase by BLM-A2 was found to be of the competitive type for the cofactor, ascorbate, and the *Ki* value, which is equivalent to the concentration of inhibitor that doubles the slope of the $1/[V]$ *versus* $1/[S]$ plots, was calculated to be 3.5×10^{-8} M. The inhibition was also found to be competitive for the substrate, tyramine, as shown in Fig. 2, and the *Ki* value was 7.7×10^{-8} M. The extent of the inhibition of BLM-A2 did not depend significantly on the fumarate concentration between 1×10^{-2} M and 5×10^{-4} M (Table 4).

Discussion

BLM derivatives were found to be a very potent inhibitor for dopamine-β-hydroxylase. They

Fig. 2. LINEWEAVER-BURK plots of tyramine concentrations against rate of hydroxylation with and without BLM-A2.

Reaction mixture contained 0.2 M sodium acetate buffer (pH 5.0), catalase (50 μg/ml), 10 mM fumarate, 10 mM ascorbate, an appropriate amount of enzyme and varying amounts for tyramine.

BLM-A2 was added without preincubation. Incubation was continued at 37°C for 30 minutes. Assay was initiated by addition of tyramine and carried out as described in Materials and Methods.

The velocities(v) are expressed as μ moles of octopamine formed from tyramine for 30 minutes. ○—○, enzyme alone; □—□, enzyme with BLM-A2 5×10^{-8} M, ●—●, enzyme with BLM-A2 8×10^{-8} M.

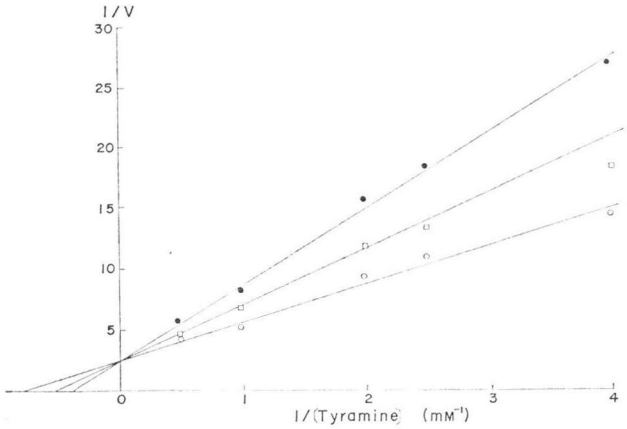

Table 5. Comparison of the kinetic properties of dopamine-β-hydroxylase inhibition by BLM, fusaric acid, glutathione and diethyldithiocarbamate.

Inhibitors	Effect on dopamine-β-hydroxylase inhibition		
	Ascorbic acid	Tyramine	Copper
BLM	competitive	competitive	reversed
Fusaric acid	competitive	uncompetitive	no effect
Glutathione and diethyldithiocarbamate	noncompetitive	noncompetitive	reversed

inhibited the enzyme at a concentration around 10^{-8} M. The most potent inhibitor of dopamine-β-hydroxylase previously reported is fusaric acid[9~11], which inhibited the enzyme at 3×10^{-8} M by 50%. Therefore, BLM has a similar inhibitory activity as fusaric acid. A substrate analogue, benzyloxyamine, inhibited the enzyme by about 60% at a concentration of 1×10^{-5} M[12,13], and the copper chelating compounds such as diethyldithiocarbamate or phenyldithiocarbamate inhibited the enzyme at a concentration between 10^{-5} M and 10^{-6} M[14]. Consequently, BLM is the most potent among the inhibitors of dopamine-β-hydroxylase which have been reported.

The mechanism of the inhibition of BLM is characteristic, since it inhibits dopamine-β-hydroxylase competitively either with ascorbic acid or tyramine. As shown in Table 5, the kinetics of BLM inhibition have characteristics of both fusaric acid and a copper-chelating agents such as diethyldithiocarbamate[14]. Since BLM readily forms a metal-complex, especially with copper, the inhibitory mechanism was initially expected to be due to chelation of the copper atom at the active site of the enzyme. However, the present experimental results indicated that the inhibition by BLM may not be due to a simple chelation. The inhibition by chelating agents such as sulfhydryl compounds is of non-competitive type with substrate and cofactor, and can be reversed by Cu^{2+} or by extensive dialysis[15]. Fusaric acid inhibits dopamine-β-hydroxylase competitively with ascorbic acid and uncompetitively with tyramine, and the inhibition is not affected by addition of copper, but is completely reversed by dialysis[9,10]. The inhibition of BLM was competitive with ascorbate and completely reversed by

dialysis, which is similar to the inhibition of fusaric acid. Interestingly, however, BLM inhibited the enzyme also competitively with the substrate, tyramine. This is different from fusaric acid but is similar to the inhibition by substrate analogues such as benzyloxyamine and benzylhydrazine[18].

It had been reported that benzyloxyamine competes initially with the substrate probably at the active site of the enzyme but once the benzyloxyamine binds to the enzyme, the inhibition will become characteristic of a noncompetitive. Therefore, after preincubation of the enzyme with benzyloxyamine, the inhibition could not be reversed by the addition of the substrate, and the activity could not be restored on extensive dialysis. On the contrary, inhibition by BLM was competitive with tyramine, and was reversed by dialysis against phosphate buffer, and preincubation did not affect the extent of the inhibition. These modes of the inhibition were different from benzyloxyamine, but were similar to those of fusaric acid.

Assuming 4 atoms of Cu(II) per 1 mol of dopamine-β-hydroxylase and the molecular weight of 300,000 daltons, 1 μg per 1 ml of pure enzyme gives a concentration of about 10^{-8} M of copper in the reaction mixture. A concentration of 10^{-7} M BLM was necessary for complete inhibition of the enzyme. Therefore, when binding of 4 moles of BLM per 1 mol of dopamine-β-hydroxylase is assumed at least a 10-fold higher concentration of BLM in the reaction mixture is required for the complete inhibition.

It can be concluded that the strong inhibition of BLM seems to be a result of chelating action of BLM with the copper firmly bound to dopamine-β-hydroxylase at the active site of the enzyme together with some other kinds of binding.

It is not likely that BLM inhibits dopamine-β-hydroxylase *in vivo*, since copper in the tissues can completely prevent the inhibition. Therefore, it seems that the contribution of the inhibitory effect of BLM on dopamine-β-hydroxylase is of little significance in the pharmacology of BLM. However, the unique inhibitory mechanism of BLM to dopamine-β-hydroxylase *in vitro* may be of significance for elucidating the mechanism of dopamine-β-hydroxylase.

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