# INHIBITION OF TYROSINE HYDROXYLASE, A Fe(II)-STIMULATED MONOOXYGENASE, BY BLEOMYCIN

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Tyrosine hydroxylase was shown to be inhibited *in vitro* by bleomycin. At a concentration of $1 \times 10^{-4}$ M, copper-free BLM-A2 inhibited tyrosine hydroxylase activity by 50%, and all other bleomycins investigated including copper-bound BLM-A2 showed similar degrees of inhibition of tyrosine hydroxylase. The kinetic data have shown that the inhibition by BLM-A2 is competitive with a tetrahydropterin cofactor (6-methyl-tetrahydropterin) and uncompetitive with tyrosine. The inhibition of tyrosine hydroxylase by bleomycin was not reversed by Fe(II) or superoxide dismutase. These results suggest that the inhibition may be neither due to the chelating action of bleomycin with ferrous ion nor due to the production of superoxide and hydroxyl radicals by the formation of an oxygen-labile Fe(II)-bleomycin complex.

Bleomycin A2 (BLM-A2), a potent antimicrobial and antitumor agent discovered by UMEZAWA, is a glycopeptide antibiotic which is isolated from *Streptomyces verticillus*[1], and has been widely used in chemotherapy of certain human tumors. BLM has a strong chelating activity with various metals, especially cupric ion, and the chemical structure of the BLM-copper complex was studied and determined by TAKITA *et al.*[2] It is known that BLM binds to DNA and causes DNA strand breakage, and in this DNA degradation reaction, ferrous ion and molecular oxygen have been proposed to serve as specific cofactors[2,3]. It is supposed that the production of hydroxyl radical or superoxide may be involved in this BLM degradation of DNA[4~8], and it is prevented by free radical scavengers[8].

Tyrosine hydroxylase [L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16. 2.] catalyzes the first step of norepinephrine biosynthesis[9], and it is well known that the enzyme is stimulated by Fe(II)[9,10]. Fe(II) chelating agents such as bipyridyl[11], *o*-phenanthroline[11] and bathophenanthroline[11], which have high affinities for divalent ion, were found to be potent inhibitors of tyrosine hydroxylase. Therefore, it would be expected that BLM can also inhibit tyrosine hydroxylase. However, the other Fe(II)-requiring dioxygenase, prolyl hydroxylase, was found to be stimulated by BLM, and the stimulation may be due to the ability of BLM to chelate Fe(II)[12]. We have therefore examined the effect of BLM on bovine adrenal tyrosine hydroxylase.

## Materials and Methods

Copper-free BLM-A2, copper-bound 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. 6-Methyl-5,6,7,8-tetrahydropterin was from Calbiochem (Los Angeles, Calif., U.S.A.); catalase from Boehringer (Mannheim, G.F.R.); and superoxide dismutase from Miles Lab. (Goodwood, S.A.). All other chemicals used were of analytical grade. Bovine adrenal glands were obtained fresh, packed in ice, from a slaughterhouse. Tyrosine hydroxylase was purified by our newly established purification

procedure about 70-fold over the soluble fraction of the adrenal medulla with 50% recovery of the activity. Details of the purification procedure will be published elsewhere. The enzyme activity was assayed by measuring L-DOPA formed from L-tyrosine as substrate by high-performance liquid chromatography[13]. The incubation mixture contained 0.2 M sodium acetate buffer (to obtain the final pH of 6.0), 0.2 mM $FeSO_4$, 0.1 M mercaptoethanol, 1.5 $\mu$g of purified tyrosine hydroxylase, 0.25 mM (or at various concentrations for kinetic studies) 6-methyltetrahydropterin ($6MPH_4$) in 0.1 M HCl, 0.05 mM (or at various concentrations for kinetic studies) L-tyrosine, and various concentrations of BLM. The molar concentration of $6MPH_4$ was estimated based on the titration by 2,6-dichlorophenol-indophenol[14] based on the molar absorbance of 18,720 $M^{-1} \cdot cm^{-1}$ at 603 nm at pH 7.18. D-Tyrosine was used for control. Incubation was carried out at 37°C for 10 minutes, and the enzymatically formed DOPA was separated by an aluminum oxide column, and measured by high-performance liquid chromatography (Yanako L-2000) with Yanako VMD-1000 voltammetric detector (Yanagimoto Manufacturing Co., Fushimi-ku, Kyoto, Japan), using a column (25 × 0.4 cm i.d.) packed with Whatman Partisil 10 ODS. The mobile phase was a 0.1 M potassium phosphate buffer (pH 2.0) with a flow-rate of 0.5 ml/min; the detector potential was set at 0.8 V against Ag/AgCl electrode. The *Km* values, maximal velocities (*V*) and *Ki* values were determined from LINEWEAVER-BURK plots[15] using WILKINSON's program[16].

## Results

### 1. Structure-Activity Relationships on the Effect of BLM on Tyrosine Hydroxylase

The effect of BLM-A2 on tyrosine hydroxylase activity is shown in Fig. 1. As seen from the figure, BLM-A2 inhibited tyrosine hydroxylase activity in the presence and absence of ferrous ion, and the inhibition of BLM-A2 was not reversed by ferrous ion. BLM concentration at 50% inhibition was about $1 \times 10^{-4}$ M in the presence of Fe(II). Table 1 shows the effect of structure of terminal amines of BLM on the inhibition. All BLM analogues including copper-bound BLM-A2 inhibited tyrosine hydroxylase activity at $1 \times 10^{-4}$ M. BLM-A5 and BLM-PEP were the most potent inhibitors, but the relationships between inhibitory activity and chemical structures at the terminal amine were not clear.

Fig. 1. Effect of BLM-A2 on tyrosine hydroxylase activity, as a function of its concentration.
Enzyme activity was assayed with standard assay conditions except that varying concentrations of BLM-A2 were added.

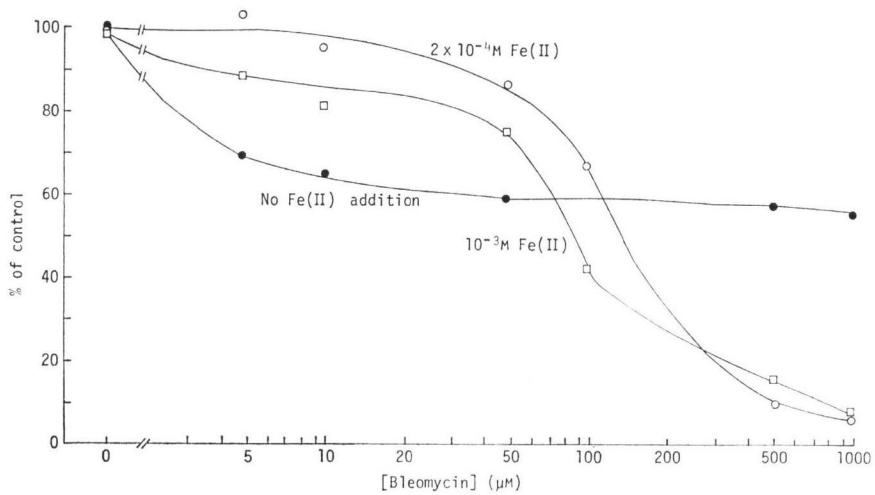


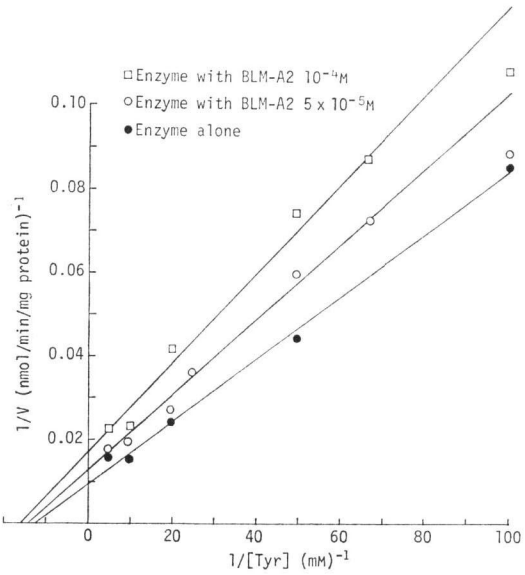
Table 1. Inhibition of tyrosine hydroxylase by bleomycins.

| Bleomycins | Tyrosine hydroxylase activity, % inhibition |
|---|---|
| Copper-free BLM-A2 | 45.2 |
| Copper-bound BLM-A2 | 57.3 |
| Deamide A2 | 66.2 |
| BLM acid | 69.3 |
| BLM-A5 | 78.6 |
| BLM-B2 | 52.8 |
| BLM-B4 | 32.5 |
| BLM-PEP | 79.6 |

BLM: Bleomycin, BLM-PEP: pepleomycin. The concentration of each BLM was $10^{-4}$ M. Enzyme activity was assayed with standard incubation mixture as described in Materials and Methods.

Fig. 2. LINEWEAVER-BURK plots of velocity *versus* substrate concentration.

Enzyme activity was assayed with the standard assay conditions as described under Materials and Methods except that varying amounts of tyrosine were added.

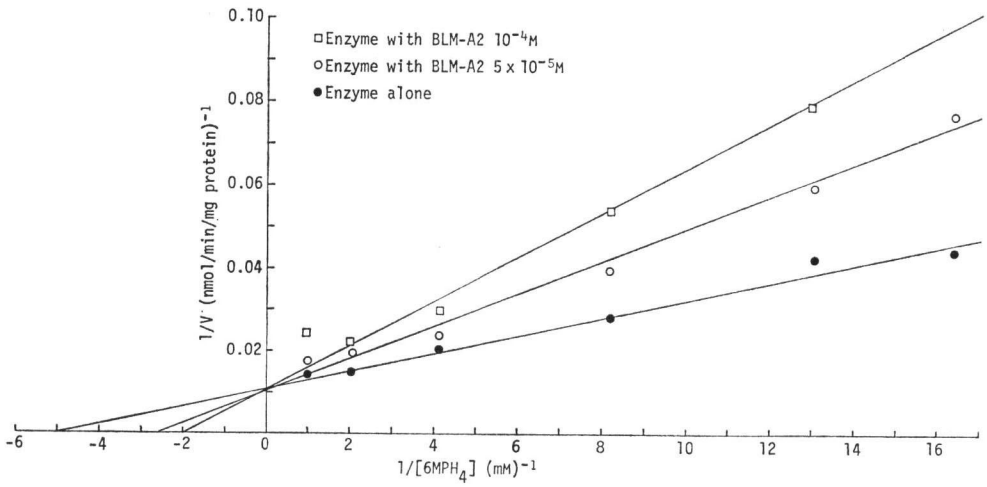



## 2. Mechanism of Inhibition

The nature of the inhibition by BLM-A2, which is the major component of natural BLM, was further investigated. The double reciprocal plots of tyrosine concentrations *versus* the rate of tyrosine hydroxylation indicate that the inhibition by BLM-A2 was uncompetitive with tyrosine over series of tyrosine concentration from $1\times10^{-5}$ M to $2\times10^{-4}$ M (Fig. 2), and the *Km* value for the tyrosine was approximately $1\times10^{-4}$ M at $2.5\times10^{-4}$ M of $6MPH_4$. LINEWEAVER-BURK plots of velocity *versus* the concentration of $6MPH_4$ are shown in Fig. 3; this inhibition was competitive with the co-factor $6MPH_4$. The *Km* value for pterin was about $2\times10^{-4}$ M. The *Ki* value of BLM-A2 was calculated to be $8.5\times10^{-5}$ M.

Fig. 3. LINEWEAVER-BURK plots of velocity *versus* 6-methyl-5,6,7,8-tetrahydropterin concentration.

Enzyme activity was assayed with the standard assay conditions as described under Materials and Methods except that varying amounts of 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$).



Instead of ferrous ion, 50 $\mu$g of catalase, which is known to stimulate tyrosine hydroxylase activity, was added to the reaction mixture. Tyrosine hydroxylase activity in the presence of catalase was 40% of that in the presence of ferrous ion, and BLM-A2 at $1\times10^{-4}$ M inhibited by 50%. Preincubation with ferrous ion and BLM-A2 for 2 minutes at 37°C had no effect on the inhibition. This inhibition was reversed by only 5% with the addition of 460 units superoxide dismutase.

## Discussion

BLM derivatives were found to be inhibitors for tyrosine hydroxylase *in vitro*. They inhibited the enzyme at a concentration around $10^{-4}$ M. Several kinds of inhibitors of tyrosine hydroxylase have been reported; tyrosine analogues such as L-$\alpha$-methyl-*p*-tyrosine[9] and 3-iodo-tyrosine[17], catechol derivatives such as catecholamines[9,17], antibiotics such as aquayamycin[18], tryptophan derivatives such as 5-hydroxytryptophan[19], chelating agents such as *o*-phenanthroline[11] and pyrroloisoxazole derivatives[20], and a new microbial inhibitor, oudenone[21]. BLM is an inhibitor as potent as catechol derivatives and has similar kinetic properties to catechols[17] or oudenone[21]; BLM inhibition is competitive toward the pterin cofactor, and uncompetitive toward tyrosine, respectively. These results suggest that BLM can combine with the enzyme or the complex of the enzyme and the pterin cofactor. The inhibition of tyrosine hydroxylase by Fe(II)-chelating agents[11] is reversed by the addition of ferrous ion, and supposed to be due to a chelation mechanism between the inhibitors and the ferrous ion, which may exist in tyrosine hydroxylase molecule[22]. BLM can form a complex with Fe(II), but since the inhibition by BLM was not reversed by the addition of ferrous ion, we can rule out the possibility of such a mechanism.

It was reported that Fe(II) and BLM system produces hydroxyl radicals or superoxide[4~8], and such radicals may also affect the enzyme activity. Radical scavengers such as catalase, ferrous ion and superoxide dismutase, added to the incubation mixture with or without preincubation, had no effect on the BLM inhibition. The results suggest that in this tyrosine hydroxylase reaction system hydroxyl radicals or superoxide are not produced because of pH, buffer and ionic strength[6]. Based on these results, it is concluded that BLM seems to directly inhibit tyrosine hydroxylase activity by a similar mechanism as oudenone[21] or catechols[17].

TAKEDA *et al.*[12] found that prolyl hydroxylase, a Fe(II)-requiring oxygenase, is stimulated by BLM and that the ability of BLM to chelate Fe(II) may be important for the stimulation of the enzyme activity. We have recently found that BLM inhibits dopamine-$\beta$-hydroxylase, a copper enzyme, due to chelating action of BLM toward the copper atom at the active site of the enzyme together with some other kinds of binding[23]. Therefore, the mechanisms of the effect of BLM *in vitro* on three oxygenases, prolyl hydroxylase, dopamine-$\beta$-hydroxylase, and tyrosine hydroxylase appear to be different. It is not likely that BLM inhibits tyrosine hydroxylase *in vivo*, since the effective concentration for the inhibition is rather high. However, BLM could be a unique chemical tool for elucidating the mechanism of oxygenase reaction.

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