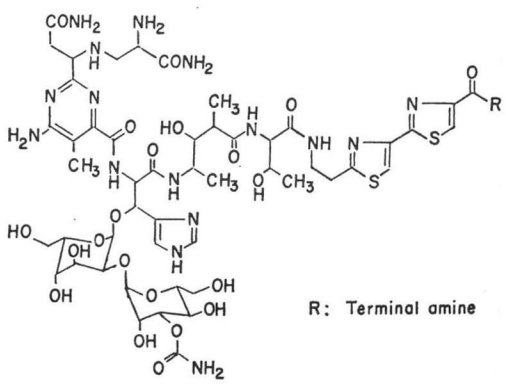
CHEMISTRY OF BLEOMYCIN. XXI METAL-COMPLEX OF BLEOMYCIN AND ITS IMPLICATION FOR THE MECHANISM OF BLEOMYCIN ACTION

Sir:

Bleomycin (BLM) is isolated from fermentation broths of *Streptomyces verticillus* as a blue powder of its equimolar Cu(II)-complex[1]. The chelated copper of BLM can be removed by precipitation with hydrogen sulfide in methanol solution. Recently, the structure of colorless metal-free BLM thus obtained was conclusively elucidated (Fig. 1)[2]. Treatment of metal-free BLM with excess amount of inorganic cupric salt such as cupric chloride in neutral aqueous solution followed by chromatographic separation on CM-Sephadex regenerates the original equimolar Cu(II)-complex in an excellent yield, although there exist many potential coordination sites in BLM.

For the structure of copper-complex of BLM, DABROWIAK *et al.* recently proposed a square-planar structure[3], which was constructed by arranging four functional groups earlier assigned by us[4] to the square-planar coordination sites using a former structure[5]. In this communication we will present three-dimensional structure of BLM Cu(II)-complex (Fig. 2), based on our own original experimental results with reference to the result of X-ray crystallographic analysis of P-3A Cu(II)-complex[6], a peptide isolated from a culture of *Streptomyces verticillus* and structurally related to BLM, and discuss implication of the metal-complex for the mechanism of BLM action.

Fig. 1. Structure of bleomycin



The copper-complex of BLM has not yet been crystallized. Therefore, the structure of the complex has been studied chemically and by spectroscopic methods. The copper-complexes of all natural BLMs give the same electronic (Fig. 3) and CD spectra[4] (see Fig. 6), which indicates that the terminal amine is not involved in the metal binding. Further, the difference UV absorption spectrum between copper-chelated and metal-free BLM (Fig. 4) is the same as that

Fig. 2. The structure of Cu(II)-complex of bleomycin

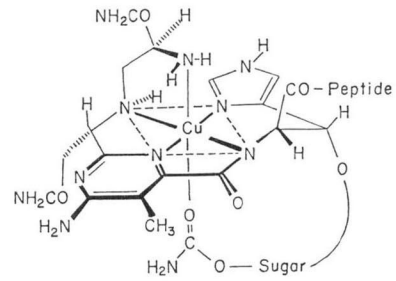


Fig. 3. Electronic absorption spectra of Cu-complexes of bleomycin and phleomycin

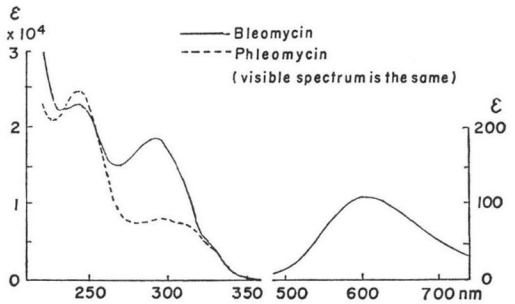
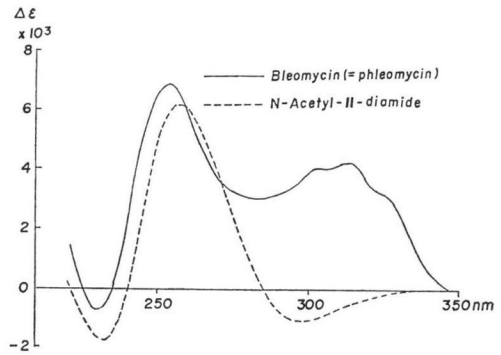


Fig. 4. Difference UV spectra of bleomycin between Cu-chelated and metal-free form, and of N-acetyl-II-diamide between 0.1 N HCl and H_2O



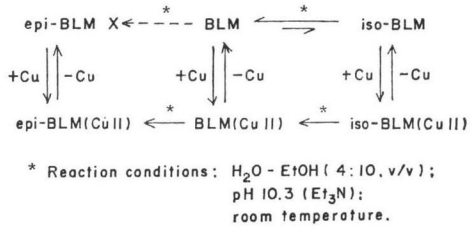
of phleomycin (PHM)[7] which indicates that the bithiazole chromophore of BLM is neither involved in the metal binding and copper-complexes of BLM and PHM have the same coordination geometry.

As reported in a previous paper[4], the α-amino group of amine component V (β-aminoalanine)[8] and the imidazole of amine component IV (β-hydroxyhistidine)[9] were suggested to be involved in the metal binding in BLM Cu-complex from the shift of their dissociation constants. That is, the pKa-values of the α-amino group and the imidazole group in metal-free BLM A2 are 7.4 and 4.7 respectively, however, in copper-chelated BLM A2 there is no ionizable group which has pKa-value between 4 and 9. Furthermore, acylation of the α-amino group of V in BLM molecule by SCHOTTEN-BAUMANN reaction under weakly basic condition is inhibited by copper-chelation, and PAULY reaction shown by metal-free BLM A2 is made negative by chelate formation as in the case of histidine. These changes in the reactivity caused by copper-chelation can be explained by involvement of the above two functional groups in the metal binding.

An extremum at 253 nm in the difference UV spectrum between copper-chelated and metal-free BLM (Fig. 4) appears to be due to formation of coordination bond between the pyrimidine and the copper. This was shown by the difference UV spectrum of a model compound for the pyrimidine chromophore, 2-(1-acetamido-2-carbamoylethyl)-6-amino-5-methylpyrimidine-4-carboxamide (abbreviated as N-acetyl-II-diamide)[2,10] (Fig. 4). That is, an extremum at 256 nm in the difference UV spectrum of the model compound between the protonated and the intact pyrimidine chromophore corresponds to the extremum at 253 nm observed in the difference absorption between the coordinated and the free pyrimidine chromophore of BLM. Incidentally, another extremum of BLM at 314 nm ($\Delta\varepsilon$ 4,300) with two inflections at the both sides is most likely due to the charge transfer transition and a weak broad asymmetric absorption centered at about 600 nm (ε 110), which is shown in Fig. 3, to the d–d transition.

Epimerization of the α-methine carbon at the pyrimidine ring-2 substituent of BLM in its copper-complex form under a mild alkaline condition also can be explained by the increased acidity of the allylic methine proton induced by

Fig. 5. Conversion of bleomycin into *iso*-bleomycin and *epi*-bleomycin.

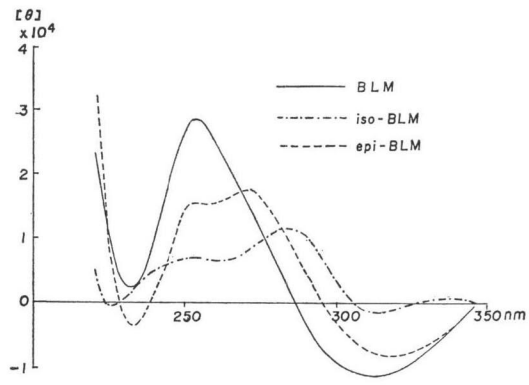


complex-formation between the pyrimidine-ring nitrogen and the metal[4]. From the steric requirement studied with space-filling molecular model, N–1, but not N–3, of the 4-aminopyrimidine chromophore and N^{π} of the imidazole were obviously found to occupy the coordination sites.

The stereomodel study also suggested that the amide nitrogen of IV is located in a favorable position for the coordination site to form 5- and 6-membered chelate rings (Fig. 2). Potentiometric titration and chromatographic behavior on CM-Sephadex of copper-complex of BLM indicated that between pH 4 and 9 a deprotonated functional group must occupy one of the coordination sites. The deprotonated amide nitrogen of IV was, therefore, inferred to be one of the coordination sites.

In the same reaction condition, under which the epimerization of copper-complex of BLM occurred, metal-free BLM gave *iso*-BLM[11] but not *epi*-BLM[4]. The isomerization occurred by migration of the carbamoyl group attached to the 3-hydroxy group of the mannose moiety to the 2-hydroxy group, and it was a reversible

Fig. 6. CD spectra of Cu-complexes of bleomycin, *iso*-bleomycin and *epi*-bleomycin.



reaction, while the epimerization of the copper-complex was irreversible (Fig. 5). A noticeable reaction was that copper-complex of *iso*-BLM was transformed to *epi*-BLM *via* BLM under the same condition as above. These results indicate that the carbamoyl group does not take part in the coordination in *iso*-BLM copper-complex but does in BLM and *epi*-BLM copper-complexes. The electronic absorption spectra of copper-complexes of BLM, *iso*-BLM and *epi*-BLM were essentially the same. However, the CD spectra were distinctly different from each other (Fig. 6). The cause of the difference of CD spectra between BLM and *epi*-BLM can be obviously understood by the difference of stereochemistry of a carbon present in a small chelate ring. The difference of CD spectra between BLM and *iso*-BLM can be explained by a strain of the chelate rings of BLM, which is caused by participation of the carbamoyl group in the coordination. If the carbamoyl group of BLM does not take part in the coordination, it will give the same CD spectrum as *iso*-BLM. When the carbamoyl group participates in the coordination in the copper-complexes of BLM and *epi*-BLM, the carbonyl oxygen, but not the deprotonated amide nitrogen, should occupy the coordination site.

At this stage in the structural study of copper-complex of BLM by chemical and spectrometric methods, the structure of P-3A copper-complex was clarified by X-ray crystallographic analysis[6]. The structure is schematically shown in Fig. 7. It has a square pyramidal coordination geometry with N^{π} and the deprotonated amide nitrogen of histidine, N-1 of the pyrimidine, and the secondary amine as the square coordination sites and the primary amine as the apical coordination site. Four ligands of P-3A Cu(II)-complex except for the secondary amine were suggested to be ligated in BLM Cu(II)-complex too as already described above. The secondary amine of BLM is also located in a favorable position for the coordination site to form additional two 5-membered chelate rings as P-3A Cu(II)-complex. Thus, BLM should have the same coordination geometry as P-3A except for the carbamoyl group at the sixth coordination site (Fig. 2). The bonding between the carbamoyl oxygen and copper appears to be very weak, because the ESR parameters of BLM and *iso*-BLM Cu(II)-complexes were almost the same (MIYOSHI *et al.*, unpublished).

HORWITZ *et al.*[12] recently reported that conversion of SV40 DNA to acid-soluble products occurred at approximately equimolar levels of Fe(II), BLM and DNA. Fe(III) did not substitute for Fe(II) in this reaction. Anaerobiosis inhibited the observed DNA degradation by BLM and Fe(II). Optical spectral studies revealed that an oxygen-labile complex was formed between BLM and Fe(II).

We observed that metal-free BLM was decomposed in the presence of Fe(II) and oxygen, but not with Fe(III). This decomposition was also

Fig. 7. The structure of Cu(II)-complex of P-3A.

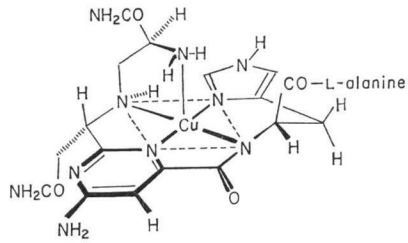
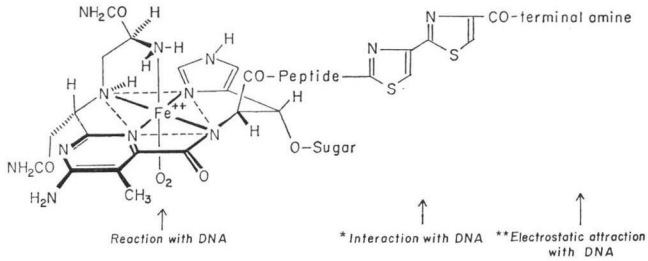


Fig. 8. The mechanism of bleomycin action.



* Interaction between BLM and DNA was suggested by M. CHIEN *et al.*[16] and us.[17]

** Electrostatic attraction between terminal amine moiety of BLM and DNA was suggested by us[17] by fluorescence spectroscopy.

caused by a catalytic amount of Fe(II) when reducing agent such as sulfhydryl compound or ascorbic acid is present. However, BLM Cu-complex was not decomposed by Fe(II) and oxygen. We isolated the major product yielded at the initial stage of the decomposition of metal-free BLM with Fe(II) and oxygen. The ^{13}C-NMR spectrum indicated that only the pseudodipeptide[2] moiety underwent the reaction of Fe(II) and oxygen and the product lacked the pyrimidine ring-2 substituent. By acid hydrolysis, the pyrimidine chromophore of this compound was completely decomposed. We are studying the structure of this reaction product. These results indicate that the active oxygen formed at the sixth coordination site of BLM Fe(II)-complex reacts with BLM itself. It is possible that the oxygen on BLM Fe(II)-complex is involved in the BLM action on DNA (Fig. 8).

If we assume that BLM Fe(II)-complex is involved in fragmentation of DNA in cells, then the following two possibilities can be proposed: The first; metal-free BLM in cells binds to DNA and thereafter Fe(II) is taken into the metal-free BLM bound to DNA. The second; BLM Cu-complex[13] reaches the nucleus and binds to DNA and thereafter the copper in the BLM Cu-complex is reductively removed[14] and replaced by Fe(II).

BLM ^{57}Co-complex has been known to bind to DNA of EHRLICH solid tumor[15]. BLM Co(III)-complex shows neither the action to inhibit the growth of cells nor to cause DNA fragmentation, although bioactive intact metal-free BLM can be regenerated chemically from the bioinactive BLM Co-complex.

Thus, studies on BLM metal-complexes will contribute to the understanding of mechanisms of cytotoxic and therapeutic action of BLM.

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