The 1.6-Å Crystal Structure of the Copper(II)-bound Bleomycin Complexed with the Bleomycin-binding Protein from Bleomycin-producing *Streptomyces verticillus*

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Bleomycin (Bm) in the culture broth of *Streptomyces verticillus* is complexed with Cu⁺⁺ (Cu(II)). In the present study, we determined the x-ray crystal structures of the Cu(II)-bound and the metal-free types of Bm at a high resolution of 1.6 and 1.8 Å, respectively, which are complexed with a Bm resistance determinant from Bm-producing *S. verticillus*, designated BLMA. In the current model of Cu(II)-Bm complexed with BLMA, two Cu(II)-Bm molecules bind to the BLMA dimer. The electron density map shows that the copper ion is clearly defined in the metal-binding domain of the Bm molecule. The metal ion is penta-coordinated by a tetragonal monopyramidal cage of nitrogens and binds to the primary amine of the β-aminoalanine moiety of Bm. The binding experiment between Bm and BLMA showed that each of the two Bm-binding pockets has a different dissociation constant ($K_d$). The $K_d$ value of 630 nM for the first Bm binding is larger than the $K_d$ value of 120 nM, indicating that the first Bm binding gives rise to a cooperative binding of the second Bm to the other pocket.

Bleomycin (Bm),¹ an anti-tumor antibiotic (Fig. 1) isolated as the Cu(II)-bound form from the culture broth of *Streptomyces verticillus* (1), shows high affinities for divalent ions such as iron, nickel, cobalt, and zinc. A family of Bm is different from one another in the terminal amine moiety. The Fe(II)-bound Bm complex, in conjunction with the reducing agent and oxygen, causes a nucleotide sequence-specific DNA cleavage (2, 3). A mechanism involving the interaction of the bithiazole moiety and the metal-binding domain of Bm with DNA has been suggested for the DNA cleavage (4–6), and the conversion of a labile Fe(II)/Bm-O₂ complex into the activated Bm can degrade DNA (2, 3). In addition, recent studies have shown that Bm can cleave the unusual tertiary structures of RNA (7, 8).

To clarify the correlations between the structure and DNA-cleaving function of the Bm molecule, it is necessary to determine the tertiary structure of Bm. However, the x-ray crystallographic analysis of Bm had not been successful until our group (9) determined the x-ray crystal structure of Bm complexed with the transposon Tn5-carried Bm-binding protein. Within the last several years, in fact, the detailed molecular interactions of Bm with DNA have been mainly studied with NMR spectroscopy (6, 10).

The antibiotic-producing microorganism must be protected from the lethal effect of its own product. We have cloned and sequenced a gene, designated *blmA*, which confers resistance to Bm, from Bm-producing *S. verticillus* (11). The gene *blmA* has been shown to encode an acidic protein with a strong affinity for Bm. The Bm-binding protein, designated BLMA, has been physicochemically characterized (12).

We have determined the crystal structure of BLMA at a high resolution of 1.5 Å by the single isomorphous replacement method including the anomalous scattering effect (13). At a 2.3-Å resolution, another group (14) determined the crystal structure of the Shble protein, which is a Bm-binding protein from taldysomycin-producing *Streptoalloteichus hindustanus* (15, 16). Both research groups have independently given a model in which the dimeric formation of the protein generates two pockets for binding to Bm.

The *Escherichia coli* transposon Tn5 carries a gene, designated *ble*, that confers resistance to Bm. The *ble* gene product, designated BLMT, has been determined to be a Bm-binding protein, like BLMA (17, 18). The amino acid sequence of BLMT shares sequence homology (21%) with BLMA. We have determined the x-ray crystal structures of BLMT uncomplexed and complexed with the metal-free Bm at 1.7- and 2.5-Å resolutions, respectively (9). Structural analysis has shown that Bm complexes with BLMT via a binding pocket formed by BLMT dimerization. Furthermore, the structural study of BLMT, complexed with the metal-free Bm, has suggested that an axial ligand for a metal ion in the Bm molecule may be the primary amine in the β-aminoalanine moiety of Bm.

In this study, because BLMTs complexed with the metal-bound Bm and the metal-free Bm were successfully crystalized, we determined the former and latter crystal structures at 1.6 and 1.8 Å, respectively. The high resolution crystallographic analysis of Cu(II)-bound Bm complexed with BLMA allowed the conclusions about the axial ligand for the metal ion. Furthermore, this high resolution analysis also showed that the occupation ratio of the cis-trans isomers with regard to the bithiazole moiety of Bm, accommodated in each of two pockets in the BLMA dimer, is different. In addition, a binding experiment in solution between metal-free Bm and BLMA demonstrated that the binding of the first Bm molecule to a pocket in
the BLMA dimer gives rise to cooperative binding of the second Bm molecule to the other pocket. We discuss the solution binding data showing cooperativity between the binding sites in light of the structural results.

**EXPERIMENTAL PROCEDURES**

**Crystal Preparation and X-ray Crystallography of BLMA Complexed with the Metal-free Bm—**BLMA, overproduced using an *E. coli* host vector system, was purified according to the methods described previously (13, 19). For the crystallization of BLMA complexed with metal-free Bm, the protein was dissolved in a 10 mM sodium phosphate buffer (pH 7.2) and incubated with a 10-fold molar excess of Bm A$_2$ (bleomycin having the γ-amino-propyl(dimethyl)sulfoxonium moiety as a terminal amine) is called bleomycin A$_2$) sulfate (a gift from Nippon Kayaku Co., Ltd.) for 1 h at room temperature. The molar ratio of Bm A$_2$ was suitable for complete binding to BLMA. Crystals of the BLMA-metal-free Bm complex were obtained by vapor diffusion at 25 °C using the hanging-drop method (20) with a precipitant solution of 17.5% (w/v) polyethylene glycol 4000 with 0.2 M ammonium acetate and 0.1 M using the hanging-drop method with the precipitant solution of 30.0% (w/v) polyethylene glycol 4000 with 0.2 M ammonium acetate and 0.1 M sodium cacodylate at pH 6.0. Data were collected on an R-AXIS IIc image plate system and processed with DENZO and SCALEPACK package (29).

Crystal Structure of the Copper(II)-Bleomycin Bound to BLMA

**FIG. 1. Structure of bleomycin A$_2$.** Wavy lines indicate the junctions between the molecular units comprising bleomycin A$_2$. In the figures, the structure is divided into four domains as follows: the DNA-binding domain (including S and B), the linker domain (including T and V), the metal-binding domain (including H, P, and A), and the sugar domain (including G and M). There are two possible planar orientations (cis and trans) of the two thiazole rings in the bithiazole moiety. Bleomycin A$_2$ has the γ-amino-propyl(dimethyl)sulfoxonium moiety as a terminal amine.

The Bm A$_2$ hydrochloride copper complex (a gift from Nippon Kayaku Co., Ltd.) for 1 h at room temperature. The molar ratio of Bm A$_2$ was suitable for complete binding to BLMA. Crystals of the BLMA-metal-free Bm crystal. The crystal structure of the BLMA-metal-free Bm complex was solved by the molecular replacement method using the programs in X-PLOR (22). The start model was a dimeric BLMA structure unconstrained with Bm, which was refined at a resolution of 1.5 Å (13). All of the model building was done on a Silicon Graphics work station with the program Xfit in the XtalView program suit (23). Refinements were initially carried out using X-PLOR with very tight non-crystallographic symmetry (NCS) restraints, followed by higher resolution refinement with SHELXL (24). At late stages of refinement, NCS restraints were relaxed as guided by the crystallographic R factor and R$_{free}$ (25) and finally ignored. The solvent molecules were positioned into sterochemically reasonable peaks using R$^{\text{c}}$-weighted Fo$-F$ and 2Fo$-F$ electron density maps. The final model, refined at a 1.8-Å resolution, had excellent geometry with no NCS restraints.

The topology and the parameter files for the Bm molecule were originally written by us in our laboratory. The parameters for the metal-binding domain, the DNA-binding domain, and the valeryl moiety in the linker domain of the Bm A$_2$ molecule were made with reference to the crystal structures of the P-3A Cu(II) complex (26), 3-(2'-phenoxy-2',4'-bithiazole-4-carboxamide)propyl dimethylsulfoxonium iodide (27), and 4-amino-3-hydroxy-2-methyl-n-valeric acid (28), respectively. The parameters for other parts (threonine in the linker domain and galose and mannose in the sugar domain) of the Bm molecule were prepared using the energy minimization structure with the program CAChE.

Crystal Preparation and X-ray Crystallography of the BLMA Cu(II)-Bm Complex—The Bm A$_2$ hydrochloride copper complex (a gift from Nippon Kayaku Co., Ltd.) was used as the Cu(II)-Bm complex. The crystals of BLMA, complexed with the Cu(II)-Bm, were obtained by vapor diffusion at 25 °C using the hanging-drop method with the precipitant solution of 30.0% (w/v) polyethylene glycol 4000 with 0.2 M ammonium acetate and 0.1 M sodium cacodylate at pH 6.0. Data were collected on an R-AXIS IIc image plate system and processed with DENZO and SCALEPACK package (29).

**TABLE I**

Statistics of data collection and refinement

| BLMA/Cu(II)-Bm | BLMA-metal-free Bm |
|---------------|-------------------|
| No. of reflections | 30,741 | 19,023 |
| Unique | 96.7 (84.5) | 80.3 (55.1) |
| Observed redundancy | 18.4 (1.98) | 10.4 (2.25) |
|Resolution range (Å) | 10.0–1.6 | 10.0–1.8 |
| R factor (%)$^a$ | 15.9 | 16.1 |
| R$_{free}$ (%)$^b$ | 20.8 | 21.9 |
| r.m.s.d. from ideality/ | 0.015 | 0.010 |
| Bonds (Å) | 0.038 | 0.032 |
| Angles (°) | 0.032 | 0.032 |

$^a$ Numbers in parentheses are values in the highest resolution shell.

$^b$ Completeness of the unique diffraction data.

$^c$ Root mean square deviation (r.m.s.d.) of bond and angle distances from ideal geometry.

The crystals belonged to the monoclinic space group P2$_1$ with unit cell dimensions a = 35.5 Å, b = 83.5 Å, c = 43.2 Å, and β = 105°. Diffraction data were collected on an R-AXIS IIc image plate system and processed with Process (21). Details of the data collection are summarized in Table I.

Crystal Structure of the Copper(II)-Bleomycin Bound to BLMA

The crystal structure of the BLMA-Cu(II)-Bleomycin complex—

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ing concentrations of Bm. This quenching is related to Trp65 in the Bm-binding pockets. Assuming that the level of fluorescence of the quenched protein, caused by the binding of the first Bm to the BLMA dimer, is the same as that by the binding of the second Bm, the quenched fluorescence must be proportional to the concentration of Bm bound to the protein. The estimated ratio of fluorescent intensity for the fully Bm-bound dimeric BLMA against the Bm-free dimeric BLMA was 0.226. Because the degree of the quenched fluorescent intensity by the dose of overexcess concentrations of Bm is not accurately obtained from the experiment, this parameter was handled as variable for the simulation of the two dissociation constants.

RESULTS AND DISCUSSION

Structure of BLMA Complexed with Cu(II)-Bm—The final 2Fo - Fc maps for the current Bm-complexed model show that the electron density is of high quality (data not shown). Like Pro residues, almost all aromatic amino acid residues have a low density hole through the center of the rings, as expected for the maps at a resolution of 1.6 Å.

Fig. 2a shows the crystal structure of BLMA complexed with Cu(II)-Bm. The topology of BLMA complexed with Bm is almost the same as that of the Bm-uncomplexed protein determined previously at a 1.5-Å resolution (13). The monomeric BLMA molecule consists of three α-helices (α1, α2, and α3) and two β-pleated sheets, in addition to an N-terminal β-strand (β1). The BLMA monomer has two very similar domains connected to the α2-helix. The N-terminal domain is composed of the α1-helix and one β-pleated sheet comprising four β-strands (β2–β5). The C-terminal domain consists of the α3-helix and one β-pleated sheet comprising five β-strands (β6–β10) (13).

The current model contains two monomeric BLMA molecules and two Cu(II)-Bm molecules in the asymmetric unit. The two monomeric BLMA molecules, related by the non-crystallographic 2-fold axis, form the dimeric structure, and a dimer accommodates two Cu(II)-Bm molecules (2:2 complex). In this study, one monomeric BLMA molecule, which takes a large conformational change at the type I β-turn composed of the residues from Ser100 to Gly103, was designated subunit A. The partner's monomer was designated subunit B. In addition, to discuss the binding mode between Bm and BLMA in detail, the protein dimer structure was divided into four regions, designated I to IV, as shown in Fig. 2a. This figure shows that regions I and III consist of the N-terminal domain of subunits A (blue) and B (red), respectively. Region II consists of the C-terminal domain of subunit A and the N-terminal β-strand of subunit B. Region IV is composed of the C-terminal domain of subunit B and the N-terminal β-strand of subunit A. The dimer form, generated by alternate arm exchange of the monomeric BLMA molecule, results in two large concavities and two grooves (Fig. 2b). Two Cu(II)-Bm molecules are accommodated in the two Bm-binding pockets. To distinguish the two Bm molecules accommodated in each of the pockets, the Bm accom-
modated between regions I and IV is designated Bm-I. The other Bm molecule, accommodated between regions II and III, is called Bm-II.

Interestingly, the electron density map of the bithiazole moiety of Bm-II shows that the orientation of the two thiazolium rings takes trans-conformation (Fig. 3b), whereas that of Bm-I shows the mixture of cis- and trans-conformations (Fig. 3c).

In many cases, the most aromatic residues are in the interior of protein. However, the long groove surface of BLMA has aromatic residues, such as Phe, His, Trp, and Met, which interacts with the N-terminal region of Bm. The groove exhibits strong hydrophobicity (Fig. 2b). The BLMA dimer has 12 Asp and 8 Glu residues. The negative charge, brought by these amino acids, is neutralized by interactions with the positively charged residues and by a positive electrostatic potential through the helix-dipole effect. However, the Asp and Glu residues are not neutralized by the interactions mentioned above. A cleft crossing vertically to the non-crystallographic 2-fold axis is observed at the interface of the BLMA dimer. The negatively charged residues, such as Asp, Glu, and His, are in the cleft (Fig. 2c).

Deviation from Symmetry in BLMA—The asymmetric unit of the Bm-uncomplexed BLMA crystal contains one subunit. The crystallographic 2-fold axis relates two subunits in a dimer. Deviation from symmetry may not be detected due to the crystallographic 2-fold symmetry (13). In contrast, the content of an asymmetric unit of the Bm-complexed BLMA crystals is a full dimer. The high resolution analysis at 1.6 Å enabled us to analyze the deviation from symmetry. Subunits A and B are aligned in a rigid body superposition to fit the main-chain atoms of each Met-Glu residue (Fig. 4). The positional root mean square deviation of the main-chain atom pairs is 0.44 Å.

Primarily, the marked conformational difference observed between both subunits is found at the type I β-turn from Ser to Gly. When compared with the Bm-uncomplexed BLMA, the type I β-turn of subunit A shifts more largely toward the bithiazole moiety of Cu(II)-Bm than that of subunit B. Another remarkable conformational difference between subunits A and B is observed at the type II' β-turn region composed of residues from Arg to Ile, which is positioned far from the Bm-binding sites. The type II' β-turn of subunit B, which interacts with the N-terminal β-strand from subunit A, takes a stable structure. However, the type II' β-turn of subunit A, having no interaction with the N-terminal β-strand of subunit B, is flexible.

Comparison of the Cu(II)-Bm-complexed and -uncomplexed Structures—BLMA complexed with Cu(II)-Bm is aligned with the protein uncomplexed with Bm using a least squares rigid body superposition (Fig. 5a). The positional root mean square deviation between both structures is 0.82 Å for the main-chain atoms. Primarily, the major conformational change that occurred by binding to Bm is observed at the type I β-turn region of each protein subunit, located from Ser to Gly (Fig. 5a). When compared with the Bm-uncomplexed BLMA, the type I β-turn in the Bm-complexed protein shifts about 5.4 Å for Pro and about 3.6 Å for Pro, respectively, toward the bithiazole moiety of Cu(II)-Bm. This conformational difference is generated by the hydrophobic interaction between the
bithiazole moiety and Pro$^{101A/B}$. Pro$^{101A}$ shifts more closely to the penultimate thiazolium ring than Pro$^{61B}$. The turn region, in the Bm-uncomplexed BLMA, is moderately flexible, whereas this region is conformationally rigid due to the binding of Bm to BLMA. In addition, the $B$ factors of the region in subunit $A$ are lower than those in subunit $B$. These observations suggest that Bm-II, accommodated in a pocket with a marked conformational change, binds more strongly to BLMA than Bm-I accommodated in the other pocket.

Because the type II $\beta$-turn composed of the residues from Arg$^{43}$ to Ile$^{46}$ in one subunit of the Bm-uncomplexed BLMA dimer interacts with the N-terminal $\beta$-strand of the subunit of the partner, the $\beta$-turn can take a stable structure. On the other hand, although the $\beta$-turn composed from Arg$^{43}$ to Ile$^{46}$ in subunit $B$ of the Bm-complexed BLMA dimer interacts with the N-terminal $\beta$-strand of subunit $A$, that in subunit $A$ has no interaction with the N-terminal $\beta$-strand of subunit $B$. This suggests that the connectivity between regions I and II is weaker than that between regions III and IV.

The two points mentioned above are local conformational changes. From the generous comparison between Bm-uncomplexed and -complexed BLMA, it is obvious that all conformational changes in the widely spread sphere are found in regions II and IV but not in regions I and III (Fig. 5b). The upper sphere in regions II and IV seems to rotate around the non-crystallographic 2-fold axis, together with the large conformational change of the type I $\beta$-turn composed from Ser$^{100A/B}$ to Gly$^{103A/B}$ (Fig. 5b).

The side chains of charged residues such as Glu$^{30}$, Arg$^{42}$, Arg$^{47}$, Glu$^{72}$, Glu$^{77}$, Arg$^{89}$, and Glu$^{93}$, located on the protein surface, give the large conformational change. Some of these changes may be affective for Bm binding. Furthermore, residues such as Lys$^{31}$, Arg$^{46}$, Ile$^{66}$, and Thr$^{119}$ are disordered in the Bm-uncomplexed and -complexed BLMA. Residue Glu$^{105}$ in the Bm-uncomplexed BLMA is also disordered, whereas Glu$^{105A/B}$ in the Bm-complexed structure is stable due to formation of hydrogen bonds with the N$\delta1$ atom of His$^{117A/B}$ and the N$\epsilon1$ atom of Trp$^{60B/A}$.

**Solution Study for Binding between Bm and BLMA**—To investigate whether each of the two Bm-binding pockets may express a different affinity for Bm, the dissociation constants of each pocket for the antibiotic in solution were measured. In this study, we defined the dissociation constants for the first and second Bm bindings to each of the two Bm-binding pockets in the BLMA dimer ((BLMA)$_2$) as $K_{d1}$ and $K_{d2}$, respectively, shown in the following Reactions 1 and 2 and Equations 1 and 2.

\[
(BLMA)_{2} + Bm \rightleftharpoons (BLMA)_{2}Bm \quad K_{d1}
\]

**REACTION 1**

\[
(BLMA)_{2}Bm + Bm \rightleftharpoons (BLMA)_{2}BmBm \quad K_{d2}
\]

**REACTION 2**

\[
K_{d1} = [Bm][BLMA]/[BLMA]_{2}Bm[BLMA] \quad \ldots (Eq. 1)
\]

\[
K_{d2} = [Bm][BLMA]_{2}Bm/[BLMA]_{2}BmBm[BLMA] \quad \ldots (Eq. 2)
\]

The $K_{d1}$ and $K_{d2}$ values obtained by the non-linear least squares method were 630 ($\pm$60) and 120 ($\pm$10) nM, respectively. Fig. 6 shows that the quenched fluorescent intensity ($\Delta F$) of the protein at the given concentrations of Bm is plotted against the logarithmic concentration of free Bm, which is not bound to the protein. The observed points are agreeably positioned on a solid line obtained by simulation. Assuming that the binding affinity of BLMA for the first Bm is the same as that for the second Bm, $K_{d1}$ must be equal to 4 times $K_{d2}$. When $K_{d1}$ and $K_{d2}$ are assumed to be half and twice the average value of $[K_{d1}]_{1/2}$, respectively, the calculated curve is expressed as a dotted line in Fig. 6. The most interesting aspect of this solution experiment is that the affinity for the second binding to the pocket of BLMA is about 20 times more favorable than that for the first binding, indicating the existence of cooperativity in the binding of Bm with BLMA.

**Interactions between Bm and BLMA**—The structure of BLMA is a dimer with two Bm-binding pockets composed of two large concavities and two long grooves. Each pocket accommodates one Bm molecule. Because the binding mode between BLMA and Bm is almost the same in each pocket, especially for the metal-binding and sugar domains of Cu(II)-Bm, the following description is mainly focused on one pocket formed between regions II and III.

The linker, metal-binding, and sugar domains of Cu(II)-Bm are buried in the large concavity (Fig. 2, b and d) and stabilized by forming a large number of hydrogen bonds with the protein atoms (Fig. 7a). The amino group, which is attached to the pyrimidine ring in the metal-binding domain of Cu(II)-Bm, interacts with the hydrogen bonds to two backbone-carbonyl oxygens from Gly$^{113A/B}$ and Thr$^{65A/B}$. The carbonyl oxygen of the pyrimidinylpropionamide moiety of Bm forms hydrogen bonds with the Tyr$^{86A}$ hydroxy group. The oxygen and nitrogen atoms of the terminal amide group of the pyrimidinylpropionamide moiety form a number of hydrogen bonds with the Ser$^{218}$ hydroxy group and the Arg$^{221}$ carbonyl oxygen, respectively. These atoms also form hydrogen bonds with the protein atoms via water molecules. However, other components of the metal-binding domain, such as $\beta$-aminoalanine and $\beta$-hydroxyhistidine, make no polar interactions with the protein atoms. In the sugar domain of Cu(II)-Bm, the mannose moiety, but not the galactose one, contributes to the binding to the protein. In detail, the nitrogen atom of the carbamoyl group, attached to the 3’-hydroxy oxygen of the mannose moiety, forms hydrogen bonds with the backbone carbonyl oxygen of the Asp$^{60A}$ and with the Asp$^{105A}$ side-chain carboxylate via Wat$^{679}$. In contrast, the oxygen atom of the carbamoyl group forms intramolecular hydrogen bonds with the amino group of the $\beta$-aminoalanine moiety of Bm. By forming the hydrogen bond, the 4’-hydroxyl group also interacts with the Arg$^{105A}$ guanidino group. The linker domain of Cu(II)-Bm, which connects the bithiazole moiety and the metal-binding domain, interacts with protein atoms through water molecules. The Glu$^{105A}$ carboxylate...
forms hydrogen bonds with the carbonyl oxygen of the threonine moiety and with the hydroxyl group of methylvalerate of Bm through Wat625.

The bithiazole moiety of Cu(II)-Bm is buried in the long groove running along the dimer interface (Fig. 2b). The terminal thiazolium ring is completely stacked with the benzene ring of Phe33A(B) (Fig. 7c). The benzene ring of Phe38A(B) interacts hydrophobically with the methylene unit extended from the penultimate thiazolium ring of Bm. In addition, the bithiazole moiety interacts hydrophobically with the type I -turn composed of the residues from Ser100B(A) to Gly103B(A) at the other side. This suggests that the dimer formation of BLMA is very important to accommodate Bm in the antibiotic-binding pockets.

Although the terminal γ-aminopropyl(dimethyl)sulfonium, which is a specific moiety for Bm A2 carrying the positive charge, is located at the end of the long groove, it has no electron density and shows high flexibility (Fig. 3a). The negatively charged cleft runs between two protein subunits in the BLMA dimer, connecting the end of the long groove of one Bm-binding pocket to that of the other Bm-binding pocket. In the binding state, the negatively charged residues in the cleft have no interactions with the positive charge of the γ-aminopropyl(dimethyl)sulfonium moiety; furthermore, this moiety of Cu(II)-Bm is not stabilized. Therefore, these negatively charged residues in the cleft appear to be necessary for the ligand recognition of the protein but not for ligand stabilization. In the current Bm-complexed BLMA, the positively charged residues, such as Arg42B(A) and Arg47B(A), are present near the positive charge of the γ-aminopropyl(dimethyl)sulfonium moiety of Bm.
By electrostatic repulsion, the side chain of Arg47B(A) shifts away from the sulfonium moiety when compared with the Bm-uncomplexed BLMA structure and forms a salt link between its guanidino group and the carboxylate of Glu67A(B). The side chain of Arg47B(A) takes a disordered structure and is observed to be unstable.

As described above, the electron density map of the bithiazole moiety of Bm-II shows that the orientation of the two thiazolium rings takes trans-conformation, whereas that of Bm-I takes two types of cis- and trans-conformations (Fig. 3, b and c). The occupancies for each conformation were gradually adjusted to yield approximately equal mean \( B \) values; the current occupancies of cis- and trans-transforms are 0.53 and 0.47, respectively. Judging from the positional relation between the negatively charged cleft and the Bm-binding pocket, the trans isomer is likely to be preferentially recognized by the BLMA dimer. Therefore, it appears that the strong Bm-binding pocket that accompanies the large conformational change accommodates Bm-II as the trans isomer, whereas the weak Bm-binding pocket inter-exchanges Bm-I between cis- and trans-conformations.

Mechanism of the Cooperative Binding of Bm—A solution study shows the cooperative binding of two Bm molecules to the BLMA dimer; in other words, it shows that the binding affinity for the first Bm is weaker than that for the second Bm. Judging from the finding that Bm-II binds to BLMA more strongly than Bm-I, the Bm molecule, accommodated first in one pocket, is concluded to be Bm-I.

When Bm-complexed BLMA is compared with Bm-uncomplexed BLMA, it is obvious that the type I \( \beta \)-turn from

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**FIG. 7. Stereo view of the binding mode between BLMA and bleomycin.**

*Figures a, b, and c show the hydrogen bonding network between Cu(II)-Bm and BLMA. Hydrogen bonds are shown using dashed lines in black and green for intermolecular and intramolecular bonds, respectively. The carbon atoms of Bm are colored light gray. Each subunit is colored a different color, i.e. subunit A (orange) and subunit B (light blue). Copper and chloride ion are shown in cyan and purple, respectively. b, hydrogen bonding network between metal-free Bm and BLMA. Color coding is as in b, c, the binding mode of Cu(II)-Bm to BLMA in the long groove. Bm-I and surrounding residues of BLMA are shown in light blue and Bm-II and its surrounding residues in brown. The bithiazole moiety is stabilized by stacking and hydrophobic interaction as described in the text.*
Ser_{100A/B} to Gly_{103A/B} and the type II $\beta$-turn from Arg$_{43A}$ to Ile$_{46A}$ exhibit large conformational changes due to the binding of Bm to BLMA (Fig. 5, a and b). The former $\beta$-turn directly contributes to the Bm binding. Although the latter $\beta$-turn is present far from the Bm-binding site, the conformational change is observed. This conformational change of the latter $\beta$-turn can be interpreted as follows. Introduction of the positively charged sulfonium moiety in Bm A$_2$ generates electrostatic repulsion against the neighboring positive charge of Arg$_{47A(B)}$. As a result, the Arg$_{47A(B)}$ side-chain shifts to neutralize the positive charge by forming a salt bridge to the Glu$_{67B(A)}$ carboxylate. Similarly, the side-chain of Arg$_{42A(B)}$ is also destabilized, and a salt bridge, formed between its guanidino group and Asp$_{32A(B)}$ carboxylate, is dissociated due to the shift of Asp$_{32A(B)}$ carboxylate toward the positively charged sulfonium moiety in Bm A$_2$. To neutralize the positive charge of the shifted Arg$_{42A(B)}$ and Arg$_{47A(B)}$ residues, the side chain of Asp$_{45A(B)}$ undergoes the conformational change (Fig. 5b).

Interestingly, the conformational change of the side chain of Asp$_{45A(B)}$ occurs asymmetrically in the BLMA dimer. The side chain of Asp$_{45A}$ shifts more noticeably than that of Asp$_{45B}$, in addition to the adequate conformational change of the main-chain atoms (Fig. 5b). The conformational change of the type II $\beta$-turn from Arg$_{43A}$ to Ile$_{46A}$ is larger than that from Arg$_{43B}$ to Ile$_{46B}$. The large positional shift gives no interaction between the $\beta$-turn from Arg$_{43A}$ to Ile$_{46A}$ and the N-terminal $\beta$-strand of subunit B and contributes to the flexibility of the type II $\beta$-turn. On the other hand, the type II $\beta$-turn from Arg$_{43B}$ to Ile$_{46B}$ interacts with the N-terminal $\beta$-strand of the subunit of the partner in the Bm-complexed BLMA, like that in the Bm-uncomplexed BLMA. Judging from these observations, the connection between regions I and II might be weaker than that between regions III and IV.

Furthermore, the structural comparison between Bm-complexed and Bm-uncomplexed BLMA makes it possible for us to suggest that the upper spheres in regions II and IV rotate around the non-crystallographic 2-fold axis, together with the large conformational change of the type I $\beta$-turn composed from Ser$_{100A/B}$ to Gly$_{103A/B}$ (Fig. 5b). The rotational change of a widely spread sphere seems to be necessary for the Bm binding, which may be stimulated by a weakness in the connection between regions I (III) and II (IV).

We propose a putative scenario that accounts for the cooperative binding of Bm to the pockets as follows. (i) When Bm-I binds to the Bm-binding pocket between regions I and IV, because the connectivity between regions III and IV is significantly strong, a significant amount of energy is necessary to cause the rotational change of region IV, which results in weak binding of Bm-I to the pocket. (ii) A positive charge of sulfonium moiety, brought by the binding of Bm-I, causes the shift of the charged residues in region I. The shift gives rise to the conformational change of the side chain of Asp$_{45A}$, which plays a role in weakening the connectivity between regions I and II. (iii) As a result, because the rotational change of region II is caused without much energy, the binding between Bm-II and the pocket may be stimulated. Therefore, the binding affinity of Bm-II is greater than that of Bm-I. The introduction of the second positive charge at the sulfonium moiety by the binding of Bm-II provokes the shifts of the charged residues near region III, except for the distant residue, Asp$_{45B}$. This may be because the electrostatic advantage accompanying the shift of Asp$_{45B}$ is not necessary for the binding of Bm-II, because a strong hydrophobic interaction is present between bithiazole moiety of Bm-II and BLMA.

**Overall Structure of Bm**—With respect to the metal-binding domain of Bm, the ligands for the metal ion bound to Bm and their chirality are still ambiguous. Most investigators agree that the equatorial ligands are the secondary amines of the $\beta$-aminoalanine moiety, the amide nitrogen of the $\beta$-hydroxypiperazine moiety, and the nitrogens of the pyrimidine and imidazole rings. However, three possibilities have been pointed out for the axial ligands as follows: (i) the primary amine of the $\beta$-aminoalanine moiety (30–32), (ii) the carbamoyl nitrogen of the mannose moiety (33, 34), or (iii) the primary amine of the $\beta$-aminoalanine moiety and the carbamoyl nitrogen of the mannose moiety (35). We have just determined the crystal structure of the metal-free Bm complexed with BLMT (9), suggesting that the axial ligand for a metal ion is the primary amine in the $\beta$-aminoalanine moiety.

In the present study, we analyzed the BLMA-Cu(II)-Bm complex. The electron density map shows that the copper ion is clearly defined in the metal-binding domain (Fig. 3e). The copper ion is penta-coordinated by a tetragonal monopyramidal cage of nitrogens. The ion binds to the primary amine of the $\beta$-aminoalanine moiety as an axial ligand, whereas the carbamoyl group of the mannose moiety takes a stable conformation by forming hydrogen bonds with the protein atoms. Judging from these experimental results, we conclude that the primary amine of the $\beta$-aminoalanine moiety is the axial ligand for the metal ion.

With regard to the DNA-binding domain in the Bm molecule, there is the question of whether the orientation of the two thiazolium rings is cis- or trans-conformation (27, 36–38). In this study, the electron density of the bithiazole moiety of Bm-II shows that the orientation is trans, whereas that of Bm-I takes both cis- and trans-conformations (Fig. 3, b and c). Co-existence of cis and trans isomers has been found in the Co(III)-Bm structure complexed with DNA (39). These results suggest that both the energy barrier and the energy difference between cis- and trans-conformations are low and that the Bm molecule present in solution takes both cis- and trans-conformations.

**Correlation between the Structure and Function of Bm**—Despite extensive research on Bm in the last 2 decades, the DNA degradation mechanism by Bm remains unclear. Our goal is to establish a model that can clearly interpret the DNA cleavage activity of Bm. Several models of the DNA-Bm complex have been constructed by NMR analysis. As an example, there is a model using the activated Bm analogue, Co(III)-Bm A$_2$, in which a hydroperoxide ion binds to the metal ion as the sixth ligand (6).

Our Cu(II)-Bm A$_2$ model, shown in Fig. 8, is aligned with the Co(III)-Bm model using the least squares rigid body superposition of atoms in the metal-binding domain. This superposition shows high similarity in the metal-binding domain; accordingly, the root mean square deviation of those atom pairs in this domain is 0.70 Å. A striking difference between both of them is observed at the linker domain. The Cu(II)-Bm model takes a more extended form in which the bithiazole moiety is far from the metal-binding domain, whereas the Co(III)-Bm takes a more compact form in which the bithiazole moiety folds back toward the metal-binding domain. This is obvious in the change of the V-CO, T-N, T-CO, T-CO dihedral angle from $\approx125^\circ$ in the BLMA-Cu(II)-Bm complex to $\approx68.5^\circ$ in the DNA-Co(III)-Bm complex. The DNA-Co(III)-Bm model has suggested that the threonine carbonyl oxygen orients itself so that it can act as a hydrogen bond acceptor for the hydroperoxide hydrogen. The DNA cleaving activity of the mutant Fe(II)-Bm, which replaced threonine in the linker domain of Bm with glycine, is much lower than that of the wild-type Fe(II)-Bm (40). Because glycine is conformationally more flexible than threonine, the conformational rigidity of the linker domain is likely
to be necessary to maintain hydroperoxide for cleaving DNA. It is well known that Cu(II)-Bm does not exhibit DNA cleaving activity (41). In this study, in fact, hydroperoxide was not observed in the electron density map of the Cu(II)-Bm model. If hydroperoxide is placed in the same position as in the DNA:Co(III)-Bm model (6), the threonine carbonyl oxygen is not able to form the hydrogen bond with the hydroperoxide hydrogen as a result of the extended conformation, which is shown in our Bm model, that is Bm needs to take a compact form to generate hydroperoxide and cleave DNA.

**Detailed Structural Differences between Cu(II)-Bm and the Metal-free Bm**—Although the overall structure of Bm complexed with BLMA is almost the same between the metal-free Bm and the Cu(II)-bound Bm, a few differences were observed and are mentioned below.

The positions of the water molecules connecting Bm to BLMA in the BLMA-Cu(II)-Bm complex are different from those in the BLMA-metal-free Bm complex (Fig. 7, a and b). The water molecules are shifted by the introduction of the chloride ion in the BLMA-Cu(II)-Bm complex. A water molecule, designated Wat569F (F denotes metal-free) in the BLMA-metal-free Bm complex, positions itself just below the putative equatorial plane of the Bm molecule and forms hydrogen bonds with the amide nitrogen atoms of the linker domain. In the BLMA-Cu(II)-Bm model, this water molecule is replaced by a chloride ion. The chloride ion makes an ionic bond with Cu(II) and forms hydrogen bonds with the amide nitrogen atoms of the linker domain of Bm. Due to the large atomic radius of the chloride ion, Wat609 shifts away from the corresponding position in the BLMA-metal-free Bm model to avoid a van der Waals violation. This causes the positional shifts of neighboring water molecules. As a result, the water molecule is not present at the position to form the hydrogen bond between the His117A imidazole and the carbonyl oxygen of the threonine moiety. Instead, the other water molecule (Wat625) is present at the position to mediate the hydrogen bond between the Glu165A carboxylate and the carbonyl oxygen of the threonine moiety.

The binding mode of the carbamoyl group, attached to the 3'-hydroxyl oxygen of the mannose moiety of Bm in the BLMA-metal-free Bm model, is different from that in the BLMA-Cu(II)-Bm model. In the former model, the nitrogen atom of the carbamoyl group forms hydrogen bonds with the Asp60A carboxylate but not with the Asp60A backbone carbonyl, as seen in the latter model. However, the oxygen atom of the carbamoyl group alternatively forms hydrogen bonds with the Asp60A backbone carbonyl through the water molecule in the former model. In the BLMA-Cu(II)-Bm model, the carbamoyl group moves to the β-aminoalanine moiety and takes a more rigid conformation with lower B factors. The position of the water molecule observed in the BLMA-metal-free Bm complex model is replaced with the oxygen atom of the carbamoyl group in the BLMA-Cu(II)-Bm model. In addition, the carbamoyl group seems to be stacked with the pyrimidine ring. These conformational changes may be derived from the structural stabilization of the β-aminoalanine moiety caused by the introduction of the copper ion, resulting in the stabilization of the neighboring mannose moiety. In this way, the metal binding and sugar domains of Bm, to which the copper ion is attached, take a more compact conformation than those of Bm without the metal ion. Therefore, the metallo-Bm seems to have a stronger affinity to BLMA than the metal-free Bm.

**Biological Meaning of BLMA in Bm-producing Microorganisms**—The phenomenon in which BLMA binds to Bm cooperatively suggested a way to explain the biological meaning of BLMA in the cells of Bm-producing microorganism. For example, it may be considered that, when the concentration of Bm inside the cells accompanied by antibiotic production is increased, the content of Bm trapped by BLMA is also increased. Thus, BLMA might be present to protect the cell from the toxicity of Bm by regulating the concentration of Bm in the cytoplasm.

The x-ray crystal structure of Cu(II)-Bm, determined under the condition complexed with a protein, shows clearly that an axial ligand of the Bm molecule for the metal ion-binding is the primary amine of the β-aminoalanine moiety. The present study is the first report that shows an x-ray crystallographic image of metallo-Bm. We have found previously that Bm-producing *S. verticillus* possesses an enzyme that acetylates Bm at the primary amine of the β-aminoalanine moiety (42). Moreover, we have shown that the N-acetylated Bm does not have DNA cleaving activity. The acetylation of Bm by the Bm N-acetylating enzyme interferes with the binding of a metal ion to the primary amine of the β-aminoalanine moiety, which may contribute to a loss of the DNA cleaving activity of Bm.

By means of an NMR study, another research group has revealed that the metal-free Bm is highly flexible (43). Interestingly, we demonstrated in this study that the tertiary structure of Bm bound to BLMA is almost the same regardless of the presence or absence of the metal ion. The binding affinity of Cu(II)-Bm for DNA has been reported to be greater than that of metal-free Bm (44). Moreover, the binding affinity of Cu(II)-Bm for DNA has been observed to be less than that of Cu(II)-Bm (44). To express DNA cleaving activity, it is proposed that the copper ion bound to Bm may be substituted by an iron ion after being incorporated into the target cells. The resulting Fe(II)-Bm complex binds O2 to generate the O2-Fe(II)-Bm complex. The complex is converted into “activated Bm” carrying hydroperoxide as the sixth ligand for the metal ion and Fe(III). The activated Bm probably releases hydroperoxide when bound to BLMA. Thus, BLMA may also contribute to the self-resistance of Bm-producing *S. verticillus* by inactivating hydroperoxide-bound Fe(III)-Bm.

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Crystal Structure of the Copper(II)-Bleomycin Bound to BLMA

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The 1.6-Å Crystal Structure of the Copper(II)-bound Bleomycin Complexed with the Bleomycin-binding Protein from Bleomycin-producing Streptomyces verticillus
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