THE NATURE OF THIOL-COMPOUNDS WHICH TRAP CUPROUS ION REDUCTIVELY LIBERATED FROM BLEOMYCIN-Cu(II) IN CELLS

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Bleomycin-Cu(II) [BLM-Cu(II)], which does not cause DNA strand breaks *in vitro*, exhibits antitumor activity *in vivo*. The copper in BLM-Cu(II) is reductively removed *in vivo*, and the liberated Cu(I) is trapped by intracellular thiol-proteins, thus yielding metal-free BLM. The bioactive species of BLM appears to be the iron-complex. For characterization of the thiol-proteins, thiol-compounds such as dithiothreitol (DTT), cysteine, metallothionein (MT) and alcohol dehydrogenase (ADH) were examined for their ability to trap Cu(I) liberated from BLM-Cu(II). BLM-Cu(II) was incubated at 37°C with the thiol-compounds anaerobically for 1 hour followed by aerobic incubation for 10 minutes. Under these conditions DTT converted BLM-Cu(II) to metal-free BLM, but cysteine did not. However, in the presence of MT, cysteine gave metal-free BLM. The Cu(I) liberated from BLM-Cu(II) by cysteine was trapped by MT with accompanying liberation of Zn(II) and Cd(II) from MT. A part of the liberated Zn(II) formed the complex with the metal-free BLM. Metal-free BLM was also formed from BLM-Cu(II) by combined treatment with cysteine and ADH. BLM-Cu(II) was aerobically incubated with cytosol of MT-induced rat liver at 37°C for 1 hour, and the mixture was analyzed with Sephadex G-75 column chromatography. The amount of copper in the MT fraction was increased concomitant with decrease of the Zn(II) and Cd(II). These results suggest that MT, ADH and other thiol-compounds, which have polythiol ligands, act as Cu(I)-trapping agents to yield metal-free BLM in cells.

Bleomycin (BLM) forms an equimolar complex with various kinds of metal ions[1]. Among these metal complexes, BLM-Cu(II) is most stable[1]. When metal-free BLM is injected to rats, most of the BLM is transformed to the Cu(II) complex in the blood[2]. BLM-iron complex causes DNA strand break *in vitro*[3~5], but BLM-Cu(II) does not[6]. However, BLM-Cu(II) cleaves DNA in cultured cells[7] and shows antitumor activity[8~12]. We have previously demonstrated that the cupirc ion of BLM-Cu(II) is reductively removed in cells to yield metal-free BLM and the liberated cuprous ion is trapped by cellular thiol-proteins[7]. The metal-free BLM thus produced in cells was suggested to exhibit cytotoxicity after binding with iron ion[13].

Metallothionein (MT) is well known as a thiol-protein which contains metal ions such as Zn(II), Cd(II) and Cu(I). These metal ions bind to MT by coordination to the cysteine residues through thiolate bonds[14]. Affinity of these metal ions in MT increases in the order of Cu(I)>Cd(II)>Zn(II), and the Zn(II) can be readily displaced by other metal ions[14]. Horse liver alcohol dehydrogenase (ADH) is a zinc protein which has two identical subunits[15]. The subunit binds with two Zn(II), one of which is present at the catalytic site and coordinates to two cysteine and one histidine residues, and the other zinc coordinates to four cysteine residues[15].

In the present study, in order to understand the nature of the cellular thiol-proteins, cysteine,

dithiothreitol (DTT), MT and ADH were examined for their ability to trap cuprous ion reductively removed from BLM-Cu(II).

Materials and Methods

Chemicals

BLM-A_2'-c copper complex [BLM-Cu(II)] (Nippon Kayaku Co., Ltd., Tokyo) was used for the experiments. L-Cysteine monohydrate was purchased from Nippon Rikagakuyakuhin Co., Tokyo, DL-DTT and MT from Sigma Chemical Co., St. Louis, Mo., U.S.A. ADH from Boehringer Mannheim GmbH., Mannheim, and $CdCl_2$ from Wako Pure Chemical Industries, Osaka.

Formation of Metal-free BLM from BLM-Cu(II) by Thiol-compounds

BLM-Cu(II) (0.2 mM) was incubated with 1 mM cysteine or 1 mM DTT in 10 mM phosphate buffer (pH 7.2) at 37°C for 1 hour. MT (300 μg/ml) and ADH (1 mg/ml) were reacted in the presence of 1 mM cysteine. Throughout incubation, the reaction mixtures were bubbled with N_2-gas to avoid autoxidation of the thiols. After the incubation, the mixtures were exposed to air with a vortex mixer and incubated further for 10 minutes at 37°C, and then applied to a column of CM-Sephadex C-25 (1×13 cm) equilibrated with 10 mM phosphate buffer (pH 7.2) to separate metal-free BLM and BLM-Cu(II) as reported previously[7]. The adsorbed metal-free BLM and BLM-Cu(II) were eluted with a linear gradient formed by 150 ml each of 10 and 50 mM phosphate buffer (pH 7.7) at a flow rate of 22 ml per hour. The eluate was collected at 10 minutes intervals. Metal-free BLM and BLM-Cu(II) eluted from the column were determined spectrophotometrically at 290 nm. Metal contents in the eluate were determined with a Hitachi atomic absorption spectrophotometer Z-8000.

Preparation of Rat Liver Cytosol Containing MT

Female 10 week-old Donryu rats (Nippon Rat Co., Tokyo) were ip injected with $CdCl_2$ at a dose of 3 mg/kg as cadmium once a day for 2 days, and thereafter at a dose of 1 mg/kg for further 4 days. Next day of the last injection, the rats were sacrificed by decaptiation, and the liver was excised. The liver was homogenized with a Kinematica Polytron homogenizer for 1 minute in 3 volumes of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.25 M glucose in an ice bath. The homogenate was centrifuged at 105,000×g for 1 hour at 0°C. The supernatant was used as MT-induced cytosol.

Cu(I)-trapping by MT-induced and Non-induced Rat Liver Cytosols

The cytosol of MT-induced or non-induced rat liver (2.5 ml) was mixed with 0.5 ml of 2 mM BLM-Cu(II) in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.25 M glucose, and incubated for 1 hour at 37°C. As the control, the cytosols were incubated in the absence of BLM-Cu(II) under the same conditions. The incubation mixture (2 ml) was applied to a column (1.84×45 cm) of Sephadex G-75 equilibrated with 1 mM Tris-HCl buffer (pH 8.6), and eluted with the same buffer at a flow rate of 3.1 ml per hour at 5°C. The eluate was collected at 30 minutes intervals, and the contents of copper, zinc and cadmium in the eluate were determined as described above.

Results

Fig. 1 shows the CM-Sephadex C-25 elution profile of the mixtures containing 0.2 mM BLM-Cu(II) and 1 mM cysteine or 1 mM DTT after anaerobic and subsequent aerobic incubations as described in Materials and Methods. Metal-free BLM was liberated by the treatment with DTT, but was not by cysteine. The amount of metal-free BLM liberated by DTT was 1.098 μmol which was 64.9% of total amount of BLM eluted. The copper removed from BLM-Cu(II) was found in the effluent fraction (data not shown).

In the presence of MT, metal-free BLM was liberated by the treatment with cysteine (Fig. 2). The amount of the liberated metal-free BLM was 70.4% (1.07 μmol) of the total BLM. Concomitant with the liberation of metal-free BLM, BLM-Zn(II) was formed and eluted in the same fractions as

Fig. 1. Formation of metal-free BLM from BLM-Cu(II) by thiol compounds.

The mixture (10 ml) containing 0.2 mM BLM-Cu(II), 1 mM cysteine or 1 mM DTT, and 10 mM phosphate buffer (pH 7.2) was incubated and applied to a column of CM-Sephadex C-25 to examine formation of metal-free BLM from BLM-Cu(II) as described in Materials and Methods.

——: DTT, ------: cysteine.

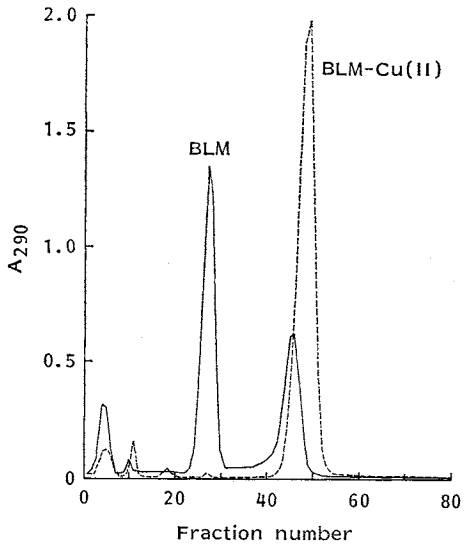
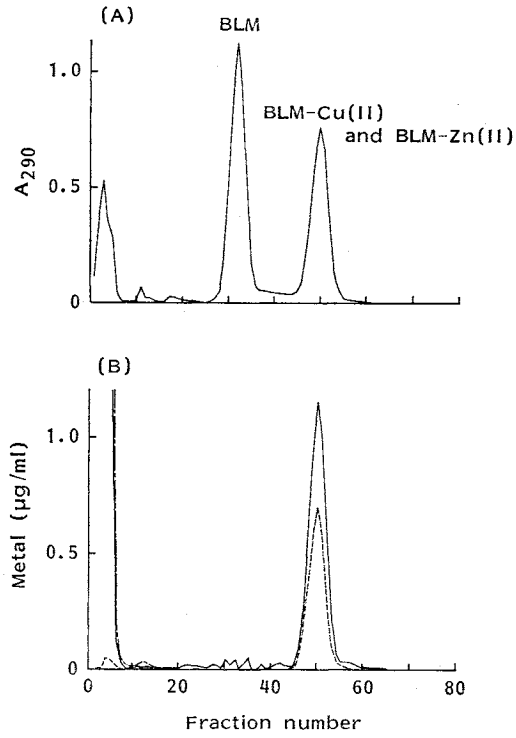


Fig. 2. Formation of metal-free BLM from BLM-Cu(II) by combined treatment with cysteine and MT.

The mixture (10 ml) containing 0.2 mM BLM-Cu(II), 300 μg/ml of MT, 1 mM cysteine and 10 mM phosphate buffer (pH 7.2) was incubated and applied to a column of CM-Sephadex C-25 to examine formation of metal-free BLM as described in Materials and Methods.

——: Cu, ------: Zn, –·–: Cd, in (B).



those of BLM-Cu(II). The amounts of BLM-Cu(II) and BLM-Zn(II) were 0.281 and 0.167 μmol, respectively. Cadmium ion was found in the effluent fraction but not in the BLM-metal complex fraction.

In order to examine the effect of cysteine in the above experiment, the mixture of 0.263 mM BLM-Cu(II) and 2 mg/ml of MT was anaerobically incubated in the presence or absence of 1 mM cysteine. The reaction mixtures were analyzed by gel-permeation HPLC (Fig. 3). In the presence of cysteine, 0.061 μmol of copper, which is 92.8% of the copper in the added BLM-Cu(II), appeared in the MT fraction, and 0.044 μmol of zinc disappeared from the MT fraction, and the corresponding amount of zinc was found in BLM fraction. On the other hand, in the absence of cysteine copper was not detected in MT fraction.

When BLM-Cu(II) was incubated with ADH in the presence of cysteine, metal-free BLM was also liberated (Fig. 4), but not in the absence of cysteine (data not shown). The amount of the liberated metal-free BLM was 0.207 μmol *i.e.* 25.7% of the total BLM eluted. The copper liberated from BLM-Cu(II) was found in the effluent. Different from the case of MT, formation of BLM-Zn(II) was not observed.

Cytosols prepared from MT-induced and non-induced rat liver were incubated with BLM-Cu(II) aerobically and analyzed with a column of Sephadex G-75. As shown in Fig. 5, when the MT-induced rat liver cytosol was incubated with BLM-Cu(II) the copper amount in the MT fraction was increased

Fig. 3. Displacement of Zn(II) of MT by Cu(I) reductively liberated from BLM-Cu(II).

The mixture (0.4 ml) containing 0.263 mM BLM-Cu(II), 2 mg/ml of MT, 1 mM cysteine and 0.1 M Tris-AcOH buffer (pH 7.2) was bubbled with N_2-gas to avoid autoxidation of cysteine, sealed in a test tube with a rubber stopper against access of air, and incubated for 1 hour at 37°C. The mixture without 1 mM cysteine was samely incubated. The incubated mixtures (0.25 ml) were next applied to a HPLC column of TSK GEL SW3000 (7.5× 600 mm, Toyo Soda Manufacturing Co., Tokyo), and eluted with 0.2 M Tris-AcOH buffer (pH 7.2) at a flow rate of 0.5 ml per minute. The eluate was collected at 2 minutes intervals. Zinc and copper contents of the eluate were determined as described in Materials and Methods.

——: Cu, ------: Zn, (A)+cysteine, (B) −cysteine.

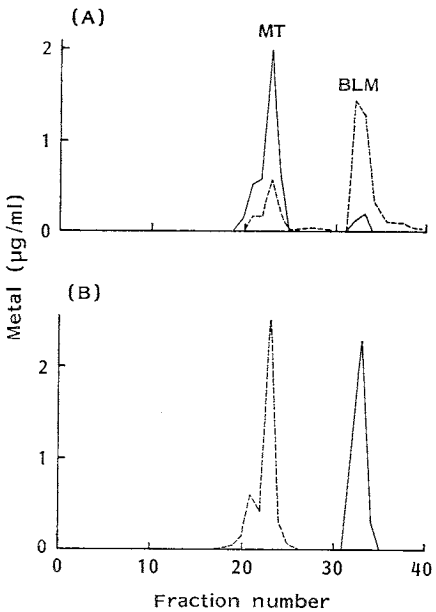
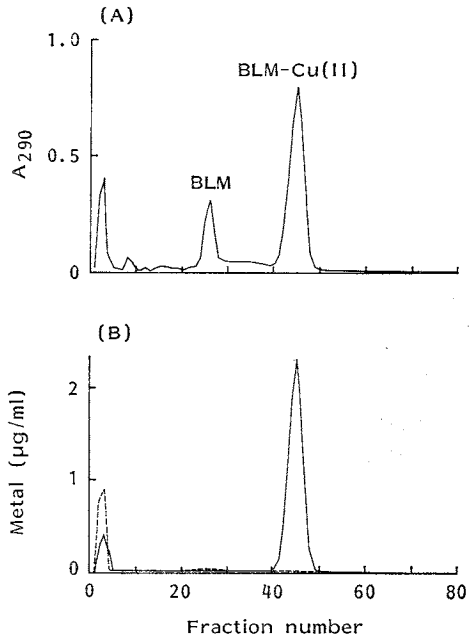


Fig. 4. Formation of metal-free BLM from BLM-Cu(II) by combined treatment with cysteine and ADH.

The mixture (5 ml) containing 0.2 mM BLM-Cu(II), 1 mg/ml of ADH, 1 mM cysteine and 10 mM phosphate buffer (pH 7.2) was incubated and applied to a column of CM-Sephadex C-25 to examine formation of metal-free BLM as described in Materials and Methods.

——: Cu, ------: Zn, in (B).



concomitant with decrease of zinc and cadmium in the MT fraction and increase of zinc in the BLM fraction. The increased amount of copper and the decreased amounts of zinc and cadmium in the MT fraction were 0.129, 0.063 and 0.014 μmol, respectively.

When the non-induced rat liver cytosol was incubated with BLM-Cu(II), the copper amount in the effluent fraction was increased concomitant with decrease of zinc in the same fraction and increase of zinc in BLM fraction (Fig. 6). The increased amount of copper and the decreased amount of zinc in the effluent fraction were 0.101 and 0.023 μmol, respectively.

Discussion

It was reported that BLM-Cu(II) was converted to metal-free BLM by cysteine under anaerobic conditions[16,17]. However, exposure to air in the above experiment caused rapid regeneration of BLM-Cu(II)[16]. The present study also showed that aeration to the anaerobic reaction mixture of BLM-Cu(II) and cysteine did not give metal-free BLM (Fig. 1). On the other hand, DTT gave metal-free BLM under the same conditions as those of cysteine (Fig. 1), though DTT is a less efficient re-

Fig. 5. Sephadex G-75 elution profile of copper, zinc and cadmium of MT-induced rat liver cytosol incubated with or without BLM-Cu(II).

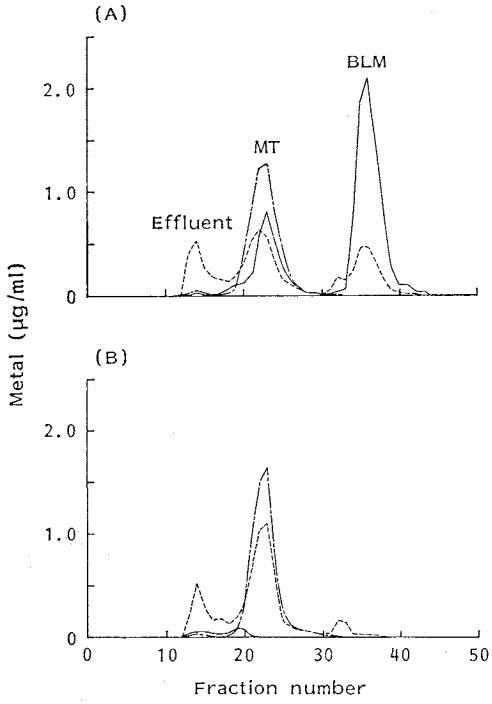
The cytosol of $CdCl_2$-injected rat liver (2.5 ml) was incubated with or without 0.5 ml of 2 mM BLM-Cu(II) and applied to a column of Sephadex G-75 to examine elution profile of copper, zinc and cadmium as described in Materials and Methods.

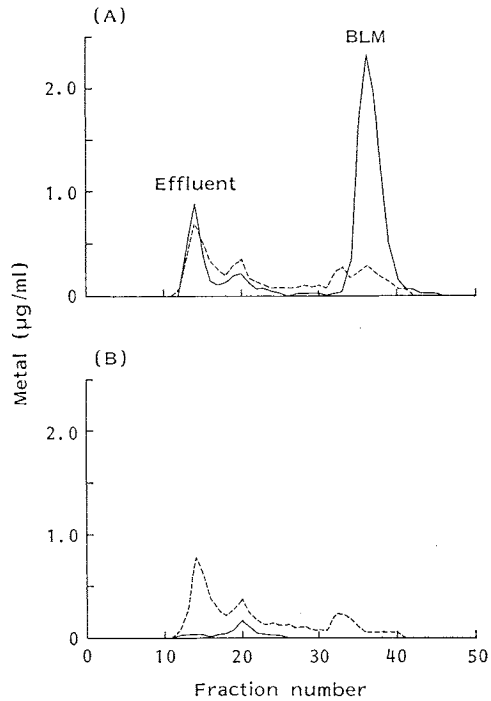
——: Cu, ------: Zn, -·-: Cd, (A)+BLM-Cu(II), (B) control.

Fig. 6. Sephadex G-75 elution profile of copper and zinc of non-induced rat liver cytosol incubated with or without BLM-Cu(II).

The cytosol of non-induced rat liver (2.5 ml) was incubated with or without 0.5 ml of 2 mM BLM-Cu(II) and applied to a column of Sephadex G-75 to examine elution profile of copper and zinc as described in Materials and Methods.

——: Cu, ------: Zn, (A)+BLM-Cu(II), (B) control.





ductant of BLM-Cu(II) than cysteine[16,17]. DTT seems to form a stable 7-membered complex with Cu(I) *via* the two thiols. The above results suggest that polythiol-proteins such as MT and ADH will trap Cu(I) liberated from BLM-Cu(II) by cysteine. This was proved by the formation of metal-free BLM in the reaction of BLM-Cu(II) with cysteine in the presence of MT or ADH (Figs. 2 and 4).

When the metal-free BLM was formed from BLM-Cu(II) in the presence of MT, the liberated Cu(I) was trapped by MT concomitant with liberation of Zn(II) from MT (Fig. 3). The amount of the copper trapped by MT was 0.061 μmol which is 1.39-fold higher than the amount of the Zn(II) liberated from MT. Similar stoichiometric relationship was obtained in the reaction of BLM-Cu(II) with MT-induced rat liver cytosol. The increased amount of copper in the MT fraction of Sephadex G-75 column chromatography was 1.68-fold of total amount of the decreased Zn(II) and Cd(II) in the same fraction. These stoichiometric relationships are consistent with the previous finding that amount of the Cu(I) which binds to MT is about 1.5-fold of total amount of the Zn(II) and Cd(II) liberated from MT[14].

A part of the liberated Zn(II) from MT formed the complex with the metal-free BLM (Fig. 2). This BLM-Zn(II) seems to be readily transformed to metal-free BLM in cells, because BLM-Zn(II) exhibits antitumor activity similar to metal-free BLM and BLM-Cu(II)[12].

When BLM-Cu(II) was incubated with MT-induced rat liver cytosol, the liberated copper appeared

only in the MT fraction, while when it was reacted with the non-induced cytosol the liberated copper appeared in the effluent fraction (Figs. 5 and 6). These results suggest that MT has higher affinity to Cu(I) than thiol-proteins in the effluent fraction.

In the reaction of BLM-Cu(II) with the non-induced rat liver cytosol, a small amount of zinc was decreased in the effluent fraction concomitant with an increase of the amount of copper in the same fraction. This result suggests that some of the proteins in the effluent fraction may be polythiol-zinc proteins.

From the results presented in this paper, MT, ADH and other thiol-compounds, which have polythiol ligands, are thought to act as Cu(I)-trapping agents to yield metal-free BLM from BLM-Cu(II) in cells.

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