STUDIES ON THE RECONSTITUTION OF MACROMOMYCIN AND AUROMOMYCIN FROM THE CHROMOPHORE AND PROTEIN MOIETIES

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The chromophores extracted from macromomycin (MCR) and auromomycin (AUR) with methanol had identical ultraviolet absorption spectra, antibacterial spectra and analytical profiles in high pressure liquid chromatography. The chromophore content of AUR was about 8 times higher than that of MCR.

MCR reconstituted from the chromophore and protein fraction was identical with native MCR by Sephadex G-50 chromatography, ultraviolet absorption spectrum and antibacterial spectrum.

The antibacterial activity of MCR and AUR was due to the chromophore; the protein moiety had no activity. However, the protein moiety enhanced the activity of the chromophore against Gram-positive bacteria, while it suppressed the activity against Gram-negative organisms. It also protected the chromophore from heat-inactivation.

Macromomycin (MCR),[1] a polypeptide antitumor antibiotic produced by *Streptomyces macromomyceticus,* inhibits the growth of Ehrlich ascites carcinoma, L1210 leukemia, P388 leukemia, B16 melanoma and Lewis lung carcinoma[2,3]. During the course of the studies on MCR, auromomycin (AUR) was isolated and crystallized[4]. AUR is yellow and inhibits the growth of both Gram-positive and Gram-negative bacteria, whereas MCR is white and inhibits the growth of only Gram-positive bacteria[4]. SUZUKI *et al.*[5,6,7] studied the mechanism of cytotoxic action of MCR and AUR which were supplied by us and reported that MCR and AUR caused DNA strand scission both *in vivo* and *in vitro*. We found that AUR contained a chromophore having a broad ultraviolet absorption maximum around 350~360 nm and AUR was converted into MCR by treatment with a column of Amberlite XAD-7.[4] The chromophore was extracted from AUR with organic solvents such as methanol or ethyl acetate, but it was very labile and purification was not successful. SUZUKI *et al.* confirmed that the methanol extract of AUR containing the chromophore caused DNA fragmentation but the chromophore-free protein did not.

As reported in a previous paper[8], we confirmed the presence of a small amount of chromophore in MCR. The chromophore fraction, which was extracted into methanol from MCR, caused DNA fragmentation and was very similar to that extracted from AUR.

In this paper, we report on the properties of MCR and AUR chromophores and the reconstitution of MCR from the chromophore and the protein fraction.

Materials and Methods

Preparation of Chromophore and Protein Moiety from MCR and AUR

Since the chromophore was extremely unstable, it was freshly prepared for each experiment and all the procedures for the preparation were carried out in a cold room in the dark.

Purified MCR or AUR powder was suspended in methanol at a concentration 1 mg/ml and stirred for an hour. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was collected and used as the chromophore fraction. If necessary, it was diluted with water or concentrated *in vacuo*.

The protein fraction precipitated by centrifugation was washed with methanol and dissolved in water. The protein solution was chromatographed on DEAE-cellulose and Sephadex G-50 in order to remove the protein denatured by methanol extraction. The purified protein moiety of MCR and AUR was lyophilized. It showed a single protein band in polyacrylamide gel electrophoresis.

The concentration of chromophore obtained from MCR and AUR is expressed as μg/ml of MCR or AUR before extraction; that is, 50 μg/ml of AUR chromophore means the chromophore obtained from 50 μg/ml of AUR. The concentration of protein is expressed as μg (dry weight)/ml.

Assay of Antibacterial Activity

The antibacterial activity of MCR and AUR was determined by the cylinder agar plate method using *Micrococcus luteus* PCI 1001 and *Escherichia coli* NIHJ as test organisms on nutrient agar plates containing 0.6% of peptone, 0.3% of yeast extract, 0.15% of beef extract and 1.0% of glucose at pH 6.7.

The antibacterial activity of the chromophore extracted from MCR or AUR was determined as described above for MCR or AUR. But in this case, plates for testing were kept in the refrigerator about 15 hours before incubation.

The minimum inhibitory concentration of MCR, AUR, their chromophores and reconstituted MCR was determined by the agar streak method using nutrient agar medium (Difco Laboratories, U.S.A.).

High Pressure Liquid Chromatography (HPLC)

A model TRI ROTAR-II High Pressure Liquid Chromatograph from Japan Spectroscopic Co., Ltd. equipped with a Uvidec 100-III and a Finepak SIL C_{18} column was used.

The chromophore extracted with methanol from MCR or AUR was concentrated to dryness *in vacuo* and redissolved in a small volume of methanol. Fifty μl of chromophore solution was injected and eluted with 60% methanol at a flow rate of 1 ml/minute. The eluate was monitored by ultraviolet absorption at 350 nm.

Reconstitution of MCR

Five ml of reaction mixture containing 10 mg of purified MCR protein moiety and the indicated amount of methanol-extracted chromophore of MCR was stirred cold for an hour in the dark. Then, the mixture was applied to a Sephadex G-50 column (18×390 nm) in order to remove the uncombined chromophore from the solution containing reconstituted MCR. Chromatography was carried out with deionized water at a flow rate of 60 ml/hour and the eluate was cut into each fractions of 2.5 ml. The effluent was monitored by absorption at 280 and 350 nm.

The reconstituted MCR fractions obtained by Sephadex G-50 column chromatography were used for the determination of the minimum inhibitory concentration and the measurement of UV-absorption spectra.

Stability of Chromophore

Three kinds of aqueous solutions, 2,000 μg/ml of MCR solution, chromophore solution derived from 2,000 μg/ml of MCR and the mixture containing chromophore fraction and protein fraction derived from 2,000 μg/ml of MCR, were used. Heat-treatment was carried out at the indicated temperatures for half an hour in the dark. UV-Irradiation by UV-lamp (Matsuda GL-10) was carried out for the indicated times at a distance of 25 cm from the lamp. For pH-treatment, sample solutions were adjusted to the indicated pH with 0.1 N HCl or 0.1 N NaOH and thereafter the solutions were left cold for an hour in the dark. After pH-treatment, an equal volume of 0.2 M phosphate buffer (pH 7.0) was added.

The antibacterial activity of treated samples was determined by the cylinder agar plate method.

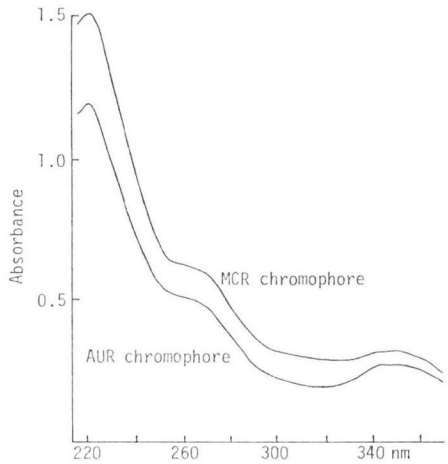
Results

Ultraviolet Absorption Spectra

As shown in Fig. 1, the ultraviolet absorption spectrum of chromophore extracted from MCR with methanol was identical to that of the AUR chromophore. It had two absorption maxima at 221 and 350 nm and a shoulder at 260 nm. The absorbance of 1 mg/ml of AUR chromophore at 221, 260 and 350 nm was equivalent to that of about 8 mg/ml of MCR chromophore.

Fig. 1. UV Absorption spectra of MCR and AUR chromophores.

AUR chromophore equivalent to 1 mg/ml of AUR in methanol and MCR chromophore equivalent to 10 mg/ml of MCR in methanol were used for the measurement.



Antibacterial Activity of Chromophore

Native AUR showed antibacterial activity against both Gram-positive and Gram-negative bacteria, whereas native MCR showed antibacterial activity only against Gram-positive bacteria as reported previously[4]. However, as shown in Tables 1 and 2, extracted MCR chromophore showed activity against not only Gram-positive bacteria but also Gram-negative bacteria and the antibacterial spectrum was similar to that of native AUR and AUR chromophore.

The protein moieties of MCR and AUR did not show any antibacterial activity even at 800

Table 1. Antibacterial spectra of MCR, MCR chromophore and MCR protein moiety.

Test organism	Minimum inhibitory concentration (μg/ml)			
	Chromophore	Protein moiety	Chromophore and protein	Native MCR
Staphylococcus aureus FDA 209P	50	>800	1.56	0.78
Staphylococcus aureus Smith	50	>800	1.56	1.56
Micrococcus flavus FDA 16	12.5	>800	0.78	0.78
Micrococcus luteus PCI 1001	25	>800	1.56	0.78
Bacillus anthracis	50	>800	1.56	1.56
Bacillus subtilis NRRL 558	100	>800	1.56	1.56
Bacillus subtilis PCI 219	100	>800	1.56	0.78
Bacillus cereus	800	>800	6.25	12.5
Escherichia coli NIHJ	400	>800	>800	>800
Proteus vulgaris OX-19	100	>800	>800	>800
Proteus rettgeri GN 311	100	>800	>800	>800
Serratia marcescens	400	>800	>800	>800
Klebsiella pneumoniae PCI 602	400	>800	>800	>800

Minimum inhibitory concentration was measured by the agar streak method. The concentration of chromophore is expressed as μg/ml of MCR before extraction.

Table 2. Antibacterial spectra of AUR, AUR chromophore and AUR protein moiety.

Test organism	Minimum inhibitory concentration (μg/ml)			
	Chromophore	Protein moiety	Chromophore and protein	Native AUR
Staphylococcus aureus FDA 209P	6.25	>800	0.1	0.1
Staphylococcus aureus Smith	12.5	>800	0.2	0.1
Micrococcus flavus FDA 16	1.56	>800	0.1	0.05
Micrococcus luteus PCI 1001	3.12	>800	0.2	0.1
Bacillus anthracis	6.25	>800	0.2	0.2
Bacillus subtilis NRRL 558	25	>800	0.78	0.2
Bacillus subtilis PCI 219	6.25	>800	0.1	0.1
Bacillus cereus	50	>800	0.39	0.2
Escherichia coli NIHJ	50	>800	>800	3.12
Proteus vulgaris OX-19	12.5	>800	>800	3.12
Proteus rettgeri GN 311	12.5	>800	>800	6.25
Serratia marcescens	50	>800	>800	50
Klebsiella pneumoniae PCI 602	50	>800	>800	6.25

Minimum inhibitory concentration was measured by the agar streak method. The concentration of chromophore is expressed as μg/ml of AUR before extraction.

μg/ml. This result suggests that the antibacterial activity of both MCR and AUR is due to its chromophore. It suggests that the antibacterial action may be due to DNA-fragmentation because the DNA-cleaving activity has been shown to be due to the chromophore[9].

The addition of the protein moiety to the chromophore gave material almost as active against Gram-positive bacteria as the native form. However, the addition of the protein moiety decreased the activity of the chromophore against Gram-negative bacteria.

Analysis of Chromophore by HPLC

HPLC analyses of chromophore from MCR and AUR are shown in Figs. 2 and 3. The absorption profile of MCR chromophore monitored at 350 nm on HPLC was almost the same as that of AUR chromophore. Only the peak eluted at 42 minutes showed antibacterial activity against both Gram-positive and Gram-negative bacteria. It also showed DNA-cleaving activity (data not shown). Two

Fig. 2. HPLC analysis of MCR chromophore.
Chromophore extracted from 30 mg of MCR was chromatographed and monitored by absorption at 350 nm.

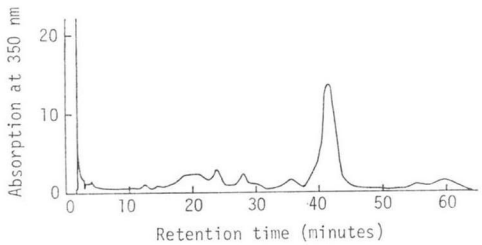
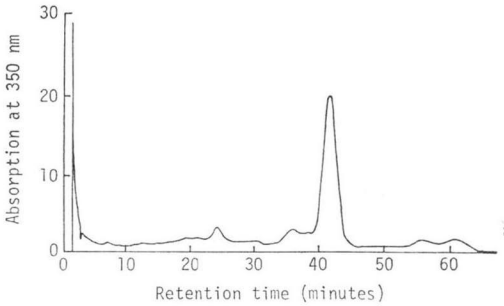


Fig. 3. HPLC analysis of AUR chromophore.
Chromophore extracted from 5 mg of AUR was chromatographed and monitored by the absorption at 350 nm.



or three other small peaks are thought to be due to the degradation of the chromophore. The results of HPLC analyses indicate that 1 mg of AUR and 8 mg of MCR contain the same amount of the chromophore.

Reconstitution of MCR from the Chromophore and Protein Moiety

The reaction mixture containing protein moiety and the indicated amount of chromophore extracted from MCR was stirred for an hour and the protein fraction recombined with chromophore was isolated by Sephadex G-50 column chromatography. As shown in Fig. 4, the higher the concentration of the chromophore in the reaction mixture, the greater the absorption of the protein fraction at 350 nm. The profiles of the Sephadex G-50 chromatography indicated that chromophore recombined with protein moiety.

Table 3 indicates the antibacterial activity of the substance reconstituted from protein moiety and varied amounts of chromophore. The substance (a) reconstituted from the reaction mixture in which the ratio of chromophore to protein moiety was the same as that in MCR showed almost the same antibacterial spectrum as native MCR. When the amount of the chromophore added to the protein moiety was increased, the antibacterial activity of the reconstituted substance against both Gram-positive and Gram-negative bacteria was enhanced and finally the substance (e) reconstituted from the reaction mixture in which the ratio of chromophore to protein moiety was 16 times higher

Fig. 4. Chromatography of reconstituted MCR on Sephadex G-50.

Mixture of 10 mg of protein moiety and chromophore equivalent to 10 (Fig. 4-a), 20 (Fig. 4-b), 40 (Fig. 4-c), 80 (Fig. 4-d) and 160 mg (Fig. 4-e) of MCR were chromatographed on Sephadex G-50. Also, the profiles of protein alone (10 mg of protein was used) and chromophore alone (equivalent to 80 mg of MCR) are shown.

——: absorption at 280 nm.

------: absorption at 350 nm.

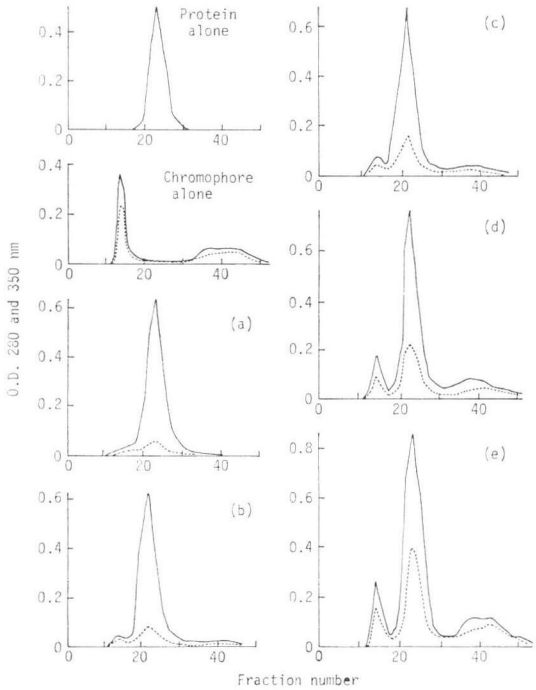


Fig. 5. UV Absorption spectra of reconstituted MCR and protein moiety.

1 mg/ml of the reconstituted substances and protein moiety were used for measurement. Sample (a), (d) and (e) were reconstituted from the reaction mixtures of 10 mg of protein moiety and chromophore equivalent to 10, 80 and 160 mg of MCR respectively. Uncombined chromophore in reconstituted substances was removed by Sephadex G-50 column chromatography.

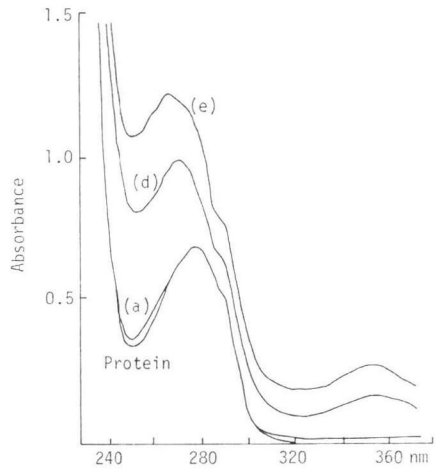


Table 3. Antibacterial spectra of MCR chromophore, MCR protein moiety, reconstituted MCR, MCR and AUR.

Test organism	Minimum inhibitory concentration (μg/ml)								
	MCR chromophore	MCR protein	(a)	(b)	(c)	(d)	(e)	MCR	AUR
Staphylococcus aureus FDA 209P	50	>800	0.39	0.39	0.39	0.39	0.2	0.78	0.1
Micrococcus flavus FDA 16	12.5	>800	0.39	0.39	0.2	0.2	0.2	0.78	0.05
Micrococcus luteus PCI 1001	25	>800	0.78	0.2	0.2	0.2	0.2	0.78	0.1
Bacillus subtilis PCI 219	100	>800	0.78	0.39	0.39	0.39	0.39	1.56	0.1
Escherichia coli NIHJ	400	>800	>800	>800	>800	50	3.12	>800	3.12
Proteus vulgaris OX-19	100	>800	>800	>800	>800	12.5	1.56	>800	3.12
Serratia marcescens	400	>800	>800	>800	>800	25	6.25	>800	25
Klebsiella pneumoniae PCI 602	400	>800	>800	>800	>800	>800	25	>800	6.25

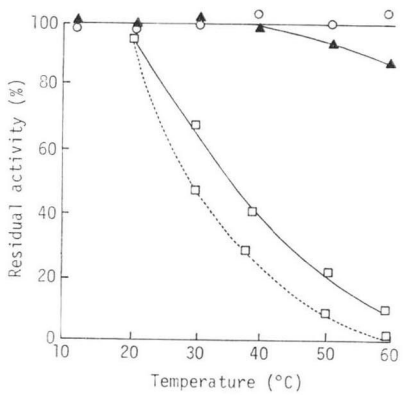
Minimum inhibitory concentration was determined by the agar streak method. Samples (a), (b), (c), (d) and (e) were reconstituted from the reaction mixtures of 10 mg of protein moiety and chromophore equivalent to 10, 20, 40, 80 and 160 mg of MCR respectively. Uncombined chromophore was eliminated by Sephadex G-50 column chromatography. The concentration of samples is expressed as μg (dry weight)/ml but the concentration of chromophore is expressed as μg/ml of MCR before extraction.

than MCR showed the same antibacterial spectrum as that of native AUR. As shown in Fig. 5, the substance (e) showed the same UV absorption spectrum as MCR[4].

Fig. 6. Stability of MCR and its chromophore to heat-treatment.

Samples were left for 30 minutes at the indicated temperature in the dark.

—: activity against *M. luteus*, ---: activity against *E. coli*, □: MCR chromophore, ○: native MCR, ▲: mixture of MCR chromophore and protein moiety.



Stability of Chromophore

As shown in Figs. 6 and 7, both MCR and AUR were relatively stable to heat-treatment but extremely labile to ultraviolet irradiation. The protein-free chromophore was much more labile than the native form. Native MCR retained all the antibacterial activity after treatment at 60°C for 30 minutes, while the activity of the chromophore was reduced to about 50% by treatment at 30~40°C for 30 minutes and was completely lost at 60°C for 30 minutes. After ultraviolet irradiation for 10~20 minutes, the activity of native MCR was reduced to about 50%. In contrast, that of the chromophore was reduced to about 10%.

As shown in Fig. 8, the stability of MCR chromophore to pH-treatment was nearly equal to that of native MCR in the range of pH 5 to 9.

Fig. 7. Stability of MCR and its chromophore to UV-treatment.

UV-Irradiation was carried out at a distance of 25 cm from UV lamp for the indicated time.

—: activity against *M. luteus*, ---: activity against *E. coli*, ○: native MCR, □: MCR chromophore, △: mixture of MCR chromophore and protein moiety.

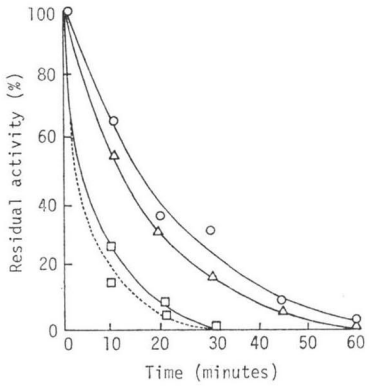
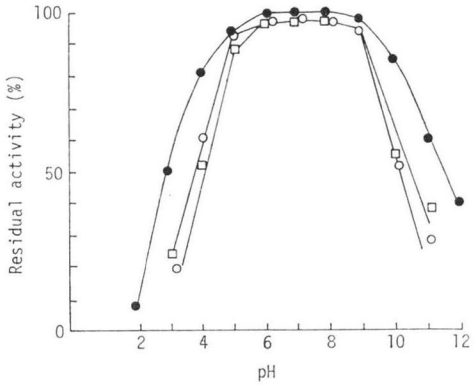


Fig. 8. Stability of MCR and its chromophore to pH-treatment.

Samples adjusted to the indicated pH were left for an hour in the dark and cold.

—: activity against *M. luteus*, ●: native MCR, □: MCR chromophore, ○: mixture of MCR chromophore and protein moiety. The activity against *E. coli* showed almost the same profile as that against *M. luteus*.



Discussion

UV Absorption spectra, antibacterial spectra and HPLC analysis of MCR and AUR chromophores indicate that MCR contains the same chromophore as AUR and MCR contains 1/8 as much chromophore as does AUR.

This conforms with the previously reported results that the DNA-cleaving activity of methanol extract of AUR was 8 to 10 times that of MCR. It also conforms with the fact that the reconstitution of AUR requires 8 times or more as much chromophore as does the reconstitution of MCR. Moreover, AUR and MCR were successfully reconstituted by varied combinations of their chromophores and protein moieties.

The reconstitution of MCR or AUR by mixing the chromophore and protein fractions suggests that the binding of the chromophore to the protein moiety may not be covalent binding.

Neocarzinostatin, another protein antitumor antibiotic, contains two kinds of biologically active chromophores[10,11] which are naphthalene carboxylic acid derivatives[11,12,13]. At the present stage, only one kind of chromophore has been found in MCR and AUR and the HPLC profiles of extracted MCR and AUR chromophores were different from that of the chromophores of neocarzinostatin[14,15].

As described in this and previous papers, the protein moiety of MCR shows neither antibacterial nor DNA-cleaving actions. The protein moiety shows a protective effect on inactivation of the chromophore and enhances the activity of the chromophore against Gram-positive bacteria but suppresses the activity against Gram-negative bacteria. The protein moiety suppresses DNA strand scission by the chromophore. Moreover, the effect of the protein moiety of MCR or AUR is specific. Other proteins such as bovine serum albumin, human albumin and the protein of neocarzinostatin can not substitute for the protein of MCR and AUR[9].

The protein moiety in MCR and AUR may play a role in the increased permeability of Gram-positive bacteria or in the decreased permeability of Gram-negative organisms. The protein moiety may also have a role in carrying the cytotoxic chromophore into carcinoma cells, preventing the degradation of chromophore and increasing the therapeutic index.

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