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Purification and Characterization of Macrolide 2′-Phosphotransferase from a Strain of *Escherichia coli* That Is Highly Resistant to Erythromycin

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Macrolide 2′-phosphotransferase [MPH(2′)] was purified 90-fold from an erythromycin-resistant strain of *Escherichia coli*, and its enzymatic properties were investigated. MPH(2′) is an inducible intracellular enzyme which showed high levels of activity with 14-member-ring macrolides and extremely low levels with 16-member-ring macrolides. The optimum pH for inactivation of oleandomycin was 8.2, and the optimum temperature of the reaction was 40°C. Enzyme activity was lost by heat treatment at 50°C for 1 min. The isoelectric point and molecular weight of the enzyme were 5.3 and 34,000, respectively. Purine nucleotides, such as GTP, ITP, and ATP, were effective as cofactors in the inactivation of macrolides. Iodine, EDTA, or divalent cations inhibited MPH(2′) activity.

In general, erythromycin is much better known for its activity against gram-positive bacteria and *Mycoplasma pneumoniae* than for its activity against gram-negative bacteria. However, in Europe, macrolides, in particular erythromycin, have been recommended for prophylaxis of septicemia in immunocompromised patients (1, 2) and for prevention of traveler's diarrhea (4). Recently, members of the family *Enterobacteriaceae* which were highly resistant to erythromycin were isolated from patients in a hematology-oncology unit (3). Colonization of the intestinal tract by members of the family *Enterobacteriaceae* that are highly resistant to erythromycin is usually associated with previous long-term oral therapy with the drug (2–4). It has been reported that high-level resistance to erythromycin in members of the family *Enterobacteriaceae* results from production of erythromycin esterases and rRNA methylases (1, 5, 6–8, 24).

We reported previously that the structure of inactivated oleandomycin generated by erythromycin-resistant (MIC, 1,600 μg/ml) *Escherichia coli* Tf481A, which was isolated from a patient in Japan in 1983, was oleandomycin 2′-phosphate (22). This paper describes the purification and characterization of a new macrolide-inactivating enzyme, macrolide 2′-phosphotransferase [MPH(2′)].

MATERIALS AND METHODS

Bacterial strains. A clinical isolate of *E. coli* Tf481A was used for determination of drug resistance and for preparation of crude extracts. *Bacillus subtilis* ATCC 6633 (20) and *Staphylococcus aureus* 209P (13) were used as test strains in microbioassays for determination of the potencies of macrolides and lincosamide antibiotics, respectively. *E. coli* K-12 W3110 Rif^r (14), which is susceptible to macrolides, was used as a reference strain.

Media. Nutrient broth (Eiken Chemical Co. Ltd., Tokyo, Japan) was used as a liquid medium, and nutrient agar was used as a solid medium.

Antibiotics and reagents. Erythromycin, oleandomycin, and spiramycin were purchased from Sigma Chemical Co., St. Louis, Mo.; and leucomycin was purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan. Erythromycin A was a gift from Shionogi & Co. Ltd., Osaka, Japan; josamycin was a gift from Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan; midecamycin was a gift from Meiji Seika Kaisha, Ltd., Tokyo, Japan; and lincomycin and clindamycin were gifts from Upjohn Pharmaceuticals Ltd., Tokyo, Japan. Pristinamycin factors I and II were gifts from the Department of Microbiology, School of Medicine, Gunma University, Gunma, Japan. Nucleoside-5′-triphosphates were purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan.

Determination of MICs. MICs of antibiotics were determined by an agar dilution method (10, 11).

Preparation of crude extract. The crude extract was prepared as described by O'Hara et al. (22, 23). Protein concentration was measured by the method of Lowry et al. (16), with bovine serum albumin as the standard.

Enzymatic inactivation of antibiotics. Enzymatic inactivation of antibiotics was performed by the modified method of Kono et al. (11, 15). Samples of 200 μl of 40 mM ATP, 25 μl of antibiotic (1 mg/ml), and 775 μl of the crude extract diluted with TMK buffer (0.06 M KCl, 0.01 M $MgCl_2$, 0.006 M 2-mercaptoethanol in 0.1 M Tris hydrochloride buffer, pH 7.8) were mixed and allowed to react at 37°C for 1 h. A 20-μl sample of the reaction mixture was spotted on a paper disk (8 m/m dia. thin; TOYO Filter Co. Ltd., Tokyo, Japan), and the disk was heated twice for 15 s each time in a microwave oven (NE-6200; National Co. Ltd., Tokyo, Japan) to stop the reaction, and the residual potency of the antibiotic was determined by microbioassay.

Release of enzyme from *E. coli* cells by osmotic shock. Release of the enzyme from *E. coli* cells by osmotic shock was performed by the method of Neu and Chou (19) with 200 ml of culture. Cells washed with 0.85% NaCl at 3°C were suspended in 16 ml of 20% sucrose–0.03 M Tris–1 mM EDTA (pH 7.3) at 21°C. After 10 min of mixing, the cells were removed by centrifugation at 0°C. The pellet of cells was suspended in 40 ml of cold water at 3°C and mixed for 10 min. The cells removed by centrifugation were disintegrated with 3.5 ml of TMK buffer by a sonicator for 3 min at 20 kHz and centrifuged at 20,000 × *g* for 30 min. The supernatant of

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sonicated cells and the sediment (cell debris) thus obtained were stored at −20°C until use. Detection of β-lactamase, used as a reference for enzymes in the periplasmic space, was performed by iodometry (21, 26) with penicillin G as the substrate.

Induction of erythromycin resistance and MPH(2'). Induction was performed by the modified method of Kono et al. (12). An overnight broth culture of *E. coli* Tf481A was diluted 10-fold with fresh broth and shaken for 2 h at 37°C. A 0.5-ml sample of the culture in the exponential growth phase was transferred to 9.5 ml of broth containing various concentrations of erythromycin and incubated at 37°C with shaking. The effect of the time of treatment with erythromycin on induction of erythromycin resistance was also investigated.

Purification of MPH(2'). Thirty milliliters of crude extract from 2.6 liters of culture treated with 100 μg of erythromycin per ml was dialyzed against TMK buffer in cellulose tubing (seamless; size 27/32; Viskase Corp., Chicago, Ill.) at 4°C overnight. Purification of MPH(2') was performed by column chromatography at 4°C on DEAE-Sephadex A-50 (2.0 by 28 cm), Sephadex G-100 (2.3 by 40 cm), and Sephadex G-75 Superfine (2.5 by 50 cm; Pharmacia Fine Chemicals, Uppsala, Sweden). Elution of proteins from columns was monitored by the A_{280} (26).

SDS-PAGE and silver staining of the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Hancock and Nikaido (9). Silver staining of the gel was performed by the method of Merril et al. (18). The marker proteins (gel filtration calibration kit; Pharmacia Fine Chemicals) were bovine serum albumin (molecular weight [mol wt], 68,000), ovalbumin (mol wt, 43,000), chymotrypsinogen A (mol wt, 25,000), and RNase A (mol wt, 13,700).

Isoelectric focusing. The isoelectric point (pI) of the enzyme was determined with an electrofocusing apparatus, FBE3000, and Pharmalyte type 3-10 (Pharmacia Fine Chemicals; 15).

RESULTS

The inducibility of MPH(2') in *E. coli* Tf481A was investigated first. The specific activities (units per milligram of protein) of crude extracts from cells treated with 0, 6.25, 12.5, 25, 50, 100, and 200 μg of erythromycin per ml for 2 h were 26, 25, 34, 57, 76, 89, and 99, respectively; and the specific activities of crude extracts from cells treated with 100 μg of erythromycin per ml for 0, 15, 30, 60, and 120 min were 25, 38, 64, 86, and 94, respectively. The specific activity of MPH(2') with induction by treatment with erythromycin (400 μg/ml) overnight was 219.5 U/mg of protein. Oleandomycin was used as the substrate for the enzymatic reaction, and it was clearly demonstrated that MPH(2') activity was inducible.

The locations of MPH(2') and β-lactamase in *E. coli* Tf481A cells were compared by the osmotic shock method (Table 1). MPH(2') was detected in the supernatant of a sonic extract of shocked cells (fraction 5) while the periplasmic enzyme β-lactamase was detected in the cold-water fraction (fraction 4). Thus, it appears that MPH(2') is an intracellular enzyme.

MPH(2') was purified by the procedures shown in Table 2. The dialyzed crude extract was applied to a column of DEAE-Sephadex A-50 equilibrated with TMK buffer. Fractions (no. 47 to 53; 10 ml of each) with macrolide-inactivating activity were lyophilized and dissolved in 10 ml of TMK buffer. The enzyme solution was applied to a column of Sephadex G-100 equilibrated and eluted with TMK buffer. Fractions (no. 15 to 20; 5 ml of each) with macrolide-inactivating activity were lyophilized. The lyophilized enzyme, dissolved in 5 ml of TMK buffer, was applied to a column of Sephadex G-75 Superfine equilibrated and eluted with TMK buffer. Fractions (no. 26 and 27; 5 ml of each) with macrolide-inactivating activity were lyophilized, and the chromatographic step with Sephadex G-75 Superfine was repeated. The fractions that contained the purified enzyme (no. 129 to 134; 1 ml of each) were pooled and stored at −20°C. In the final step, the elution profile of the activity of the macrolide-inactivating enzyme was coincident with the A_{280} profile. The specific activity of the enzyme and the results of SDS-PAGE at various stages of purification are shown in Table 2 and Fig. 1, respectively. Purified MPH(2') showed only one band (mol wt, 34,000) on a silver-stained gel (Fig. 1, lane D). Furthermore, the band of purified protein after SDS-PAGE had the same mobility as the specific protein induced by erythromycin (Fig. 1, lanes E and F).

TABLE 1. Liberation of MPH(2') from *E. coli* Tf481A by osmotic shock

Fraction	Enzymatic activity (%)	
	MPH(2')	β-Lactamase
1[a] (Supernatant of whole culture)	0	7
2 (0.85% NaCl solution)	0	18
3 (Sucrose-Tris-EDTA solution)	0	12
4[b] (Cold water)	0	100[c]
5[d] (Supernatant of sonicated cells)	100[e]	10
6 (Cell debris)	0	0

[a] Extracellular enzyme fraction.
[b] Periplasmic-space enzyme fraction.
[c] Seventy-seven units. One unit of β-lactamase is defined as the amount of enzyme that hydrolyzes 1 μmol of penicillin G in 1 h at 37°C and pH 7.0.
[d] Intracellular enzyme fraction.
[e] Seven hundred twenty-six units.

The enzymatic properties of purified MPH(2') from *E. coli* Tf481A are shown in Table 3. With respect to its substrate specificity, purified MPH(2') showed high levels of activity with 14-member-ring macrolides and extremely low levels with 16-member-ring macrolides. The substrate specificity of purified MPH(2') for antibiotics was very similar to that of the crude extract. The specificity of the purified enzyme for nucleoside-5'-triphosphate cofactors was investigated. All of the nucleoside-5'-triphosphates tested could function as cofactors in these reactions, but the rates of reactions with purine nucleotides were higher than those of reactions with pyrimidine nucleotides.

The effects of inhibitors and ions on the activity of

TABLE 2. Summary of purification of MPH(2')

Stage (procedure)	U (10^3) of enzymatic activity (%) recovered	Sp act (U/mg of protein)	Fold purification
1 (Crude extract, dialyzed)	254.1 (100)	96.8	1
2 (DEAE-Sephadex A-50)[a]	107.3 (42.2)	1,392.2	14.4
3 (Sephadex G-100)[b]	53.3 (21.2)	2,343.0	24.2
4 (Sephadex G-75 Superfine)[b]	27.3 (10.7)	8,746.0	90.4

[a] Eluted with 800 ml of a linear gradient of NaCl (0 to 0.5 M) in TMK buffer.
[b] Eluted with TMK buffer.

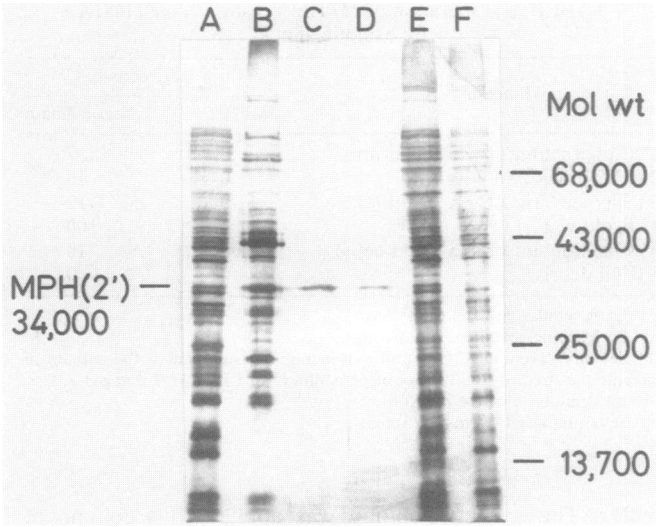

FIG. 1. Results of SDS-PAGE of MPH(2') at various stages (see Table 2) of purification. Lanes: A, stage 1; B, stage 2; C, stage 3; D, stage 4 [purified MPH(2')]; E, crude extract (induced cells); F, crude extract (noninduced cells).

MPH(2') were also investigated (Table 4). *p*-Chloromercuribenzoate at 0.1 mM and urea at 1 mM failed to affect MPH(2'). Iodine, EDTA, and the divalent cations Zn^{2+}, Fe^{2+}, Cu^{2+}, and Co^{2+} at 1 mM exhibited strong inhibitory effects on the activity of MPH(2'). In contrast, the enzymatic activity of MPH(2') was not influenced by the monovalent cations and anions tested.

E. coli Tf481A was also resistant to streptomycin, kanamycin, gentamicin, chloramphenicol, ampicillin, and sulfonamide but not erythromycin. The erythromycin resistance of strain Tf481A was not transferable to erythromycin-susceptible *E. coli* W3110 Rif^r. However, the erythromycin resistance of strain Tf481A was eliminated by treatment with ethidium bromide at 42°C.

DISCUSSION

The macrolide resistance pattern of *E. coli* Tf481A resembles that of clinical isolate BM2195, which produces erythromycin esterase (1). The inducible erythromycin resistance of strain Tf481A is due to inducible intracellular MPH(2').

For substrate specificity, MPH(2') appears to preferentially inactivate 14-member-ring macrolides rather than 16-member-ring macrolides. It was suggested that MPH(2') reacts with the 2'-OH of a desosamine moiety with the 14-member lactone aglycone but only slightly with the 2'-OH of a mycaminosylmycarose disaccharide moiety with the 16-member lactone aglycone.

Phosphorylation of oleandomycin by MPH(2') from *E. coli* Tf481A occurred at the 2'-OH of the D-desosamine moiety (21). Purified MPH(2') did not inactivate aminoglycoside antibiotics that contain desosaminelike amino sugar moieties, such as kanamycins A, B, and C; deoxykanamycin B; gentamicins C_1, C_{1a}, and C_2; sisomicin; tobramycin; amikacin; habekacin; netilmicin; ribostamycin; and neomycin B.

The inhibitory effect of EDTA, divalent cations, or iodine on the enzymatic activity of MPH(2') might suggest that Mg^{2+} is essential to the reaction of MPH(2') and that the active site of MPH(2') is related to amino acids such as Phe, Tyr, Trp, and His.

The enzymatic properties of MPH(2') from *E. coli* Tf481A were remarkably different from those of MPH(2') from *Streptomyces coelicolor* (17, 25) with regard to optimum pH, substrate specificity, and relative stimulation of the reaction by various cofactors.

An analysis of the MPH(2')-producing gene of *E. coli* Tf481A is in progress.

TABLE 3. Enzymatic properties of purified MPH(2')[a]

Drug	Substrate specificity (%)
Erythromycin A[b]	87
Oleandomycin[b]	100
Midecamycin[c]	3
Spectinomycin[c]	5
Lencomycin[c]	1
Josamycin[c]	1
Lincomycin[d]	0
Clindamycin[d]	0
Pristinamycin factor I[e]	0
Pristinamycin factor II[e]	0

[a] The optimum temperature was 40°C, the optimum pH was 8.2, the optimum pI was 5.3, and the mol wt was 34,000. The rates of inactivation of macrolides with the purine cofactors ATP, GTP, and ITP were 100 (reference), 934, and 970%, respectively; and the rates of inactivation of macrolides with the pyrimidine cofactors CTP, TTP, and UTP were 24, 19, and 36%, respectively.
[b] Fourteen-member-ring macrolide.
[c] Sixteen-member-ring macrolide.
[d] Lincosamide.
[e] Streptogramin.

TABLE 4. Effects of inhibitors and ions on enzymatic activities of MPH(2')[a]

Inhibitor or salt[b]	Concn (mM)	% Inhibition
p-Chloromercuribenzoic acid	1.0	—[c]
Urea	1.0	—
	50.0	14
I_2	0.1	30
	1.0	100
EDTA	10.0	100
$ZnSO_4$	1.0	93
$FeSO_4$	1.0	66
$CuSO_4$	1.0	52
$CoSO_4$	1.0	51

[a] The enzyme preparation was incubated in the presence or absence of each inhibitor or ion at the concentrations indicated for 3 min at 37°C and then assayed for enzyme activity, which was determined on oleandomycin by bioassay.
[b] Salts that had no effect on the enzyme were NaCl, CH_3COONa, Na_2HPO_4, KNO_3, K_2SO_4, $(NH_4)_2SO_4$, and Na_2SO_4.
[c] —, Effect of less than ±10% of enzyme activity (defined as no effect).

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