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Purification of macrolide 2′-phosphotransferase from *Streptomyces coelicolor* Müller

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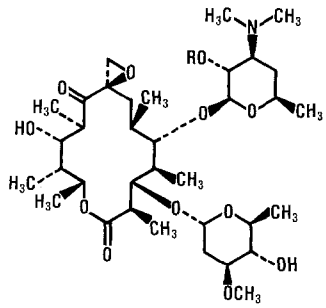
SUMMARY

An enzyme that catalyzes 2′-*O*-phosphorylation of oleandomycin and several other macrolide antibiotics has been purified approximately 47-fold from cell-free extracts of *Streptomyces coelicolor* Müller, NRRL 3532 (UC® 5240). The reaction product was verified as being oleandomycin-2′-*O*-phosphate by mass spectrometry. As a result of purification, the enzyme was separated from two lincosaminide inactivating enzyme activities also present in the cell-free extract.

INTRODUCTION

Fermentations and cell-free preparations of *Streptomyces coelicolor* Müller, NRRL 3532 (UC 5240) were reported to bring about inactivation of oleandomycin, tylosin, spiramycin, leucomycin and erythromycin by 2′-*O*-phosphorylation (Fig. 1) [7,12]. This enzymatically catalyzed process was shown to require ATP or UTP, and Mg2+, Ca2+, Co2+, Zn2+, or Mn2+ [8,12]. *S. coelicolor* Müller in fermentation also converts lincosaminide antibiotics to mixtures of 3-(5′-ribonucleotides) and 3-*O*-phosphates [1,2]. As might be expected, cell-free preparations of this organism also catalyze the formation of both types of these lincosaminide inactivation products. However, the conversions are pH dependent with phosphorylation occurring optimally near pH 8.5, and ribonucleotidylation near pH 6.0 [4,6]. Substrate, coenzyme and divalent metal cation requirements indicated that these 3 antibiotic inactivating reactions were catalyzed by different enzymes in the *S. coelicolor* Müller cell-free extract [8].



1a R=H

1b R=P(OH)$_2$

Fig. 1. The structures of oleandomycin (1a) and oleandomycin-2′-*O*-phosphate (1b).

A similar, but not identical, macrolide 2′-phosphotransferase was discovered and purified from a strain of *Escherichia coli* found highly resistant to erythromycin [9–11]. However, this enzyme differs somewhat from the streptomycete enzyme in terms of substrate, coenzyme and divalent metal cation specificities, as well as pH optimum [8,12].

MATERIALS AND METHODS

S. coelicolor Müller was maintained, grown and harvested as described previously [6,7,12]. The cell-free extract was prepared using lysozyme by the method of Hey and Elbein [5] as described in our earlier publications [6,12]. The resulting cell-free extract was treated with manganese chloride to remove nucleic acids, and was then subjected to ammonium sulfate fractionation exactly as described by Coats and Argoudelis [4] in the purification of the lincosaminide phosphorylating enzyme.

The precipitate obtained by treatment with $(NH_4)_2SO_4$ between 30 and 60% saturation was dissolved in 100 mM

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potassium phosphate, pH 7.0 and dialyzed vs. 10 mM Tris-HCl, pH 8.0 overnight. Particulate material was removed by centrifugation at $10^4 \times g$ for 1 h. The supernatant fluid was loaded onto a FLPC Mono Q anion exchange column (HR 5/5, Pharmacia) and equilibrated with 10 mM Tris-HCl, pH 8.0. Bound proteins were eluted with a linear gradient of 0 to 300 mM NaCl in 10 mM Tris-HCl, pH 8.0 for 30 min. The absorbance of the eluent was monitored continuously at 280 nm. Flow rate was 1 ml/min, and 1 ml fractions were collected. Macrolide 2′-phosphotransferase activity was found in fractions 22–24. Enzyme protein was quantitated by the Bio-Rad method which is based on the procedure of Bradford [3].

Enzymic phosphorylation of oleandomycin was performed using reaction volumes ranging from 2 to 20 ml. The reaction mixtures contained oleandomycin, 50–150 μg; ATP (Sigma, grade II), 40 μmol; cell-free extract, 500–700 μg or purified enzyme, 40–60 μg; $MgCl_2$, 40 μmol and potassium phosphate, 5 μmol per ml of distilled water. These mixtures were adjusted to pH 7.2 and held at 28 °C for 24 to 36 h. Enzymic phosphorylation and nucleotidylation of pirlimycin were performed as above with pirlimycin at a concentration of 250 μg/ml and with Tris buffer at 10 μmol/ml in the case of pirlimycin phosphorylation. The reaction mixtures were adjusted to pH 8.5 and 6.3 with NaOH and HCl, respectively, and incubated as before.

A biological assay employing *Micrococcus luteus*, UC 130 was used to quantitate these inactivations. One biounit of anti- *M. luteus* activity was defined as the amount of antibiotic that when applied to a 12.7 mm paper disc (Schleicher and Schull, No. 740-E) produced a zone of growth inhibition of 20 mm on a seeded agar culture. Through comparison of experimental biounit activities to those of standard antibiotic solutions, μg values were assessed.

A 24 h reaction mixture containing oleandomycin, 150 μg; 47-fold purified macrolide 2′-phosphotransferase, 40 μg; $MgCl_2$, 40 μmol; ATP, 40 μmol and potassium phosphate, 5 μmol per ml of distilled water was lyophilized. Twenty mg of the lyophilized reaction mixture was dissolved in 1 ml of distilled water and passed over a 500 mg C_{18} column (Analtech). The column was washed with 3 × 2 ml of distilled water. The organic material was then eluted with 2 × 1 ml of methanol, and the methanolic solution was blown dry under a stream of N_2. The sample was dissolved in 2-hydroxyethyl disulfide matrix and was analyzed by Fast Atom Bombardment Mass Spectrometry (FAB-MS) using Xenon atoms of 8 kV energy. The mass spectrum was recorded on a V.G. ZAB 2F high resolution mass spectrometer.

RESULTS AND DISCUSSION

Macrolide 2′-phosphotransferase was purified from cell-free extracts of *S. coelicolor* Müller as described earlier in the text of this report. Table 1 summarizes this procedure which resulted in ca. 47-fold purification of the enzyme when oleandomycin was employed as assay substrate. Using this procedure about 1/3 of the activity units in the cell-free extract were present in the pooled column fractions. The larger number of activity units present in the resuspended $(NH_4)_2SO_4$ precipitate may reflect the removal of an enzyme(s) competing for ATP.

The oleandomycin conversion product formed using the 47-fold purified enzyme in the presence of ATP and Mg2+ was determined to be oleandomycin-2′-*O*-phosphate by mass spectrometry. The positive ion FAB mass spectrum of the isolated sample showed pseudomolecular ions at *m/z* 790 and 812 for oleandomycin-2′-*O*-phosphate and at *m/z* 688 and 710 for oleandomycin (Table 2).

The results of our previous studies indicated that macrolide 2′-phosphotransferase was not the same enzyme as those catalyzing lincosaminide phosphorylation and nucleotidylation [8]. Table 3 presents data showing that the *S. coelicolor* Müller cell-free extract catalyzes macrolide and lincosaminide inactivation by the 3 reactions indicated. However, pooled fractions from a Mono Q anion exchange column are able to catalyze only oleandomycin phosphorylation. Data presented here prove the macrolide 2′-phosphotransferase to be separated and dis-

TABLE 1

Purification of macrolide 2′-phosphotransferase

Purification stage	Protein (mg)	Activity units[a]	Specific activity[b]	Fold purification
Cell-free extract	681	2898	4.3	–
$(NH_4)_2SO_4$ precipitate (30–60% sat.)	241	3506	14.6	3.4
Active mono Q fractions	4.6	920	200	46.5

[a] 1 unit = 1 μg oleandomycin inactivated per hour.

[b] Specific activity = units per mg protein.

TABLE 2

FAB-MS data for oleandomycin-2′-*O*-phosphate

Compound	Formula	MW	Observed mass (m/z)
Oleandomycin	$C_{35}H_{61}NO_{12}$	687	–
(M + H)	$C_{35}H_{62}NO_{12}$	688	688
(M + Na)	$C_{35}H_{61}NNaO_{12}$	710	710
Oleandomycin-2′-*O*-phosphate	$C_{35}H_{62}NO_{15}P$	767	–
(M + Na)	$C_{35}H_{62}NNaO_{15}P$	790	790
(M + 2Na-H)	$C_{35}H_{61}NNa_2O_{15}P$	812	812

TABLE 3

Conversion of oleandomycin and pirlimycin catalyzed by cell-free extract and purified macrolide 2′-phosphotransferase from *S. coelicolor* Müller

Enzyme source	Reaction assayed (units/mg protein)		
	Oleandomycin phosphorylation	Pirlimycin phosphorylation	Pirlimycin nucleotidylation
Cell-free extract	10.3	13.1	27.1
Active mono Q fractions	333.3	0	0

Crude and purified enzymes were prepared separately from those described in Table 1.

tinct from the lincosaminide inactivating enzymes present in the cell-free extract.

REFERENCES

1 Argoudelis, A.D. and J.H. Coats. 1971. Microbial transformation of antibiotics. V. Clindamycin ribonucleotides. J. Am. Chem. Soc. 93: 534–535.

2 Argoudelis, A.D., J.H. Coats and S.A. Mizsak. 1977. Microbial transformations of antibiotics. Clindamycin ribonucleotides. J. Antibiot. 30: 474–487.

3 Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of proteins-dye binding. Anal. Biochem. 72: 248–254.

4 Coats, J.H. and A.D. Argoudelis. 1971. Microbial transformation of antibiotics: Phosphorylation of clindamycin by *Streptomyces coelicolor* Müller. J. Bacteriol. 108: 459–464.

5 Hey, A.E. and A.D. Elbein. 1968. Partial purification and properties of a trehalase from *Streptomyces hygroscopicus*. J. Bacteriol. 96: 105–110.

6 Marshall, V.P., T.E. Patt and A.D. Argoudelis. 1986. Enzymic nucleotidylation of lincosaminide antibiotics. J. Indust. Microbiol. 1: 17–21.

7 Marshall, V.P., J.I. Ciadella, L. Baczynskyj, W.F. Liggett and R.A. Johnson. 1989. Microbial O-phosphorylation of macrolide antibiotics. J. Antibiot. 42: 132–134.

8 Marshall, V.P., W.F. Liggett and J.I. Ciadella. 1989. Enzymic inactivation of lincosaminide and macrolide antibiotics: Divalent metal cation and coenzyme specificities. J. Antibiot. 42: 826–830.

9 O'Hara, K., T. Kanda and M. Kono. 1988. Structure of a phosphorylated derivative of oleandomycin, obtained by reaction of oleandomycin with an extract of an erythromycin-resistant strain of *Escherichia coli*. J. Antibiot. 41: 823–827.

10 O'Hara, K., T. Kanda, K. Ohmiya, T. Ebisu and M. Kono. 1989. Purification and characterization of macrolide 2′-phosphotransferase from a strain of *Escherichia coli* that is highly resistant to erythromycin. Antimicrob. Agents Chemother. 33: 1354–1357.

11 O'Hara, K., T. Kanda, K. Ohmiya, T. Ebisu and M. Kono. 1989. Purification and characterization of macrolide-2′-phosphotransferase from a strain of *Escherichia coli* that is highly resistant to erythromycin. Antimicrob. Agents Chemother. 33: 2025.

12 Wiley, P.F., L. Baczynskyj, L.A. Dolak, J.I. Cialdella and V.P. Marshall. 1987. Enzymic phosphorylation of macrolide antibiotics. J. Antibiot. 40: 195–201.