PURIFICATION OF LINCOSAMINIDE *O*-NUCLEOTIDYLTRANSFERASE FROM *Streptomyces coelicolor* MÜLLER

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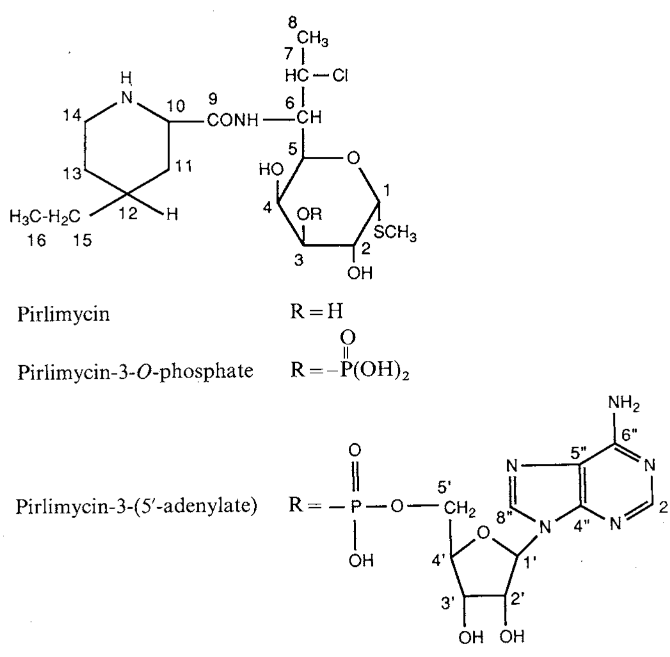
An enzyme (lincosaminide *O*-nucleotidyltransferase) that catalyzes 3-(5'-ribonucleotidylation) of pirlimycin and several other lincosaminide antibiotics has been purified approximately 35-fold from cell-free extracts of *Streptomyces coelicolor* Müller NRRL 3532 (UC 5240). The crude enzyme was prepared using lysozyme and was treated with $MnCl_2$ and $(NH_4)_2SO_4$. Final purification was achieved by anion exchange chromatography. The pirlimycin reaction product was verified as being pirlimycin-3-(5'-adenylate) by NMR spectroscopy and MS. As a result of purification, this lincosaminide nucleotidylating and inactivating enzyme was separated from the macrolide phosphorylating enzyme also present in the cell-free extract.

Fermentations and cell-free preparations of *Streptomyces coelicolor* Müller were reported to bring about inactivation of pirlimycin, lincomycin, and clindamycin through 3-(5'-ribonucleotidylation) (Fig. 1).[1,2] This enzymatically catalyzed process was shown to require ATP, ITP, GTP, CTP, or UTP,[2] and Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, or Mn^{2+}.[2,3] *S. coelicolor* Müller also converted these lincosaminides to their 3-*O*-phosphates (Fig. 1); however, the conversions were pH dependent with ribonucleotidylation occurring

Fig. 1. Structures of pirlimycin, pirlimycin-3-(5'-adenylate), and pirlimycin-3-*O*-phosphate.



optimally near pH 6.0, and phosphorylation near pH 8.5.[2,3] Cell-free extracts were prepared using lysozyme.[4] In addition, *S. coelicolor* Müller has been reported to bring about 2'-*O*-phosphorylation of oleandomycin, tylosin, spiramycin, leucomycin, and erythromycin.[3,5~7] Substrate, coenzyme and divalent metal cation requirements,[3] as well as purification data,[7] indicated that these three antibiotic inactivating reactions are catalyzed by different enzymes in the *S. coelicolor* Müller cell-free extract.

Although inactivation of lincosaminide antibiotics *via* 3-(5'-ribonucleotidylation) was first observed in *S. coelicolor* Müller, this phenomenon has now been reported to occur in lincosaminide-resistant Staphylococci.[8] Interestingly, the purified staphylococcal enzyme catalyzed the conversion of lincomycin to its 3-(5'-ribonucleotide) and clindamycin to its 4-(5'-ribonucleotide).

Materials and Methods

Microbiological and Enzyme Purification Methods

S. coelicolor Müller NRRL 3532 (UC 5240), was grown as described in two of our previous publications,[2,5] and was harvested by centrifugation in the cold at $10^4 \times g$ for 15 minutes. Sedimented mycelial mass (100 g) was suspended in 100 ml of 100 mM potassium phosphate, pH 7.0, and was centrifuged as described. The washed mycelia were then resuspended in 100 ml of 10 mM potassium phosphate, pH 7.5, which contained EDTA at 500 mg/liter, and were lysed by egg white lysozyme (Sigma, grade I) using the procedure of HEY and ELBEIN.[4] 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[9] in the purification of the lincosaminide phosphorylating enzyme. The precipitate obtained by treatment with ammonium sulfate between 30 and 60% saturation was dissolved in 100 mM potassium phosphate, pH 7.0, and dialyzed vs. 10 mM potassium phosphate pH 8.0 overnight. Particulate material was removed by centrifugation at $10^4 \times g$ for 1 hour. The supernatant fluid was loaded onto a fast protein liquid chromatography (FPLC) Mono Q anion exchange column (HR 5/5, Pharmacia) equilibrated with 20 mM potassium phosphate, pH 8.0. Bound proteins were eluted with a linear gradient of 0 to 300 mM KCl in 10 mM potassium phosphate, pH 7.4, for 30 minutes. The absorbance of the eluent was monitored continuously at 280 nm. Flow rate was 1 ml/minute, and 1 ml fractions were collected. Nucleotidyltransferase activity was present in fractions 9~11 (40~60 mM KCl). Enzyme protein was quantitated by the Bio-Rad method which is based on the procedure of BRADFORD.[10]

Bioassay Methods

Enzymic nucleotidylation of pirlimycin was performed using reaction volumes ranging from 2 to 20 ml. The reaction mixtures contained pirlimycin 250 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 6.3 and were held at 28°C for 1 to 36 hours. Enzymic phosphorylation of oleandomycin was performed as above with oleandomycin at a concentration of 150 μg/ml. The reaction mixtures were incubated at pH 7.2 as indicated above.

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.

MS Methods

A 24-hour reaction mixture containing purified enzyme 2.4 mg, ATP 800 μmol, $MgCl_2$ 800 μmol, pirlimycin 5 mg, and potassium phosphate 100 μmol added per 20 ml of distilled water was lyophilized. Twenty mg of the lyophilized material was dissolved in 1.0 ml of distilled water and was applied to a C18 Bondelute (Analytichem International) column. This column was washed three times with 2.0 ml of distilled water. The organic material was eluted twice with 1.0 ml of methanol. The methanolic solution was

evaporated under a stream of nitrogen. The solid residue was then dissolved in 100 μl of CH_3OH, and a fraction of this was applied to the FAB probe tip which contained the matrix 2-hydroxyethyldisulfide (2-HED). The samples were analyzed by FAB-MS using xenon atoms of 8 KV energy. The mass spectra were recorded on the VG ZAB-2F HR mass spectrometer employing the UPACS II data system.

Purification Methods

Samples of the pirlimycin reaction mixture were combined to give 1.31 g of crude material. Upon dissolving in 15 ml water, the combined sample was loaded onto a 4 × 30 cm column of Sephadex G-10 (Pharmacia, Piscataway, NJ) equilibrated with water. The column was eluted with water, and 20 ml fractions were collected. HPLC was used to determine fractions containing the pirlimycin inactivation product. A pool of the selected fractions was made and concentrated to dryness. Upon redissolving the concentrated sample in a small amount of water, a white precipitate formed. The supernatant was siphoned off and refrigerated overnight at 4°C resulting in the formation of additional precipitate. NMR analysis of the precipitate indicated it to be pure pirlimycin-3-(5′-adenylate).

HPLC Methods

HPLC was performed with a Dynamax C-18 cartridge column on a Varian Star 9020 system employing a Varian 9065 diode array detector. Samples were injected with a Waters 715 Ultrawisp autosampler and eluted with acetonitrile and 10 mM sodium phosphate adjusted to pH 3 with concentrated phosphoric acid. A gradient from 100% to 25% phosphoric acid over 20 minutes followed by an isocratic elution at the final combination for 5 minutes was used to separate the components of interest. In this system, authentic pirlimycin-3-(5′-adenylate)[11] eluted at 11.2 minutes, while authentic pirlimycin-3-*O*-phosphate[9] eluted at 11.5 minutes. Use of the Star data station in combination with the diode array detector enabled the positive identification of these components in injected samples.

NMR Methods

1D and 2D ^1H and ^{13}C NMR spectra were recorded on a Bruker AM-300 Spectrometer operated at 300 MHz and 75 MHz, respectively. Spectra were run in perdeuterated DMSO. Standard pulse sequences were used to obtain these spectra. A 1-second delay time was inserted between pulses to obtain the ^{13}C NMR spectrum, while a 2-second delay was inserted to obtain the ^1H NMR spectrum. For ^1H-^1H COSY spectrum, a total of 128 experiments with 32 scans per experiment were performed and zero filled to 1/2 K. For ^1H-^{13}C correlation spectrum, a total of 128 experiments were performed with 512 scans per experiment and zero filled to 1/2 K.

Results and Discussion

The lincosaminide *O*-nucleotidyltransferase was purified from cell-free extracts of *S. coelicolor* Müller as described earlier in this report. Table 1 summarizes these procedures which resulted in *ca.* 35-fold purification of the enzyme when pirlimycin was employed as the assay substrate. Using this procedure, about 1/3 of the activity units contained in the cell-free extract were present in the pooled column fractions (9 ~ 11).

Table 1. Purification of lincosaminide *O*-nucleotidyltransferase.

Purification stage	Total protein (mg)	Activity units[a]	Specific activity[b]	Fold purification
Cell-free extract	578	14,713	26	—
$(NH_4)_2SO_4$ precipitate (30 ~ 60%)	300	13,971	47	1.8
Active Mono Q fractions	6	5,250	875	33.7

[a] 1 unit: 1 μg of pirlimycin inactivated/hour.
[b] Specific activity: units/mg protein.

Table 2. Conversions of pirlimycin and oleandomycin catalyzed by enzymic preparations of *Streptomyces coelicolor* Müller.

Enzyme source	Reaction assayed (units/mg protein)	
	Pirlimycin ribonucleotidylation	Oleandomycin phosphorylation
Cell-free extract	26	9.1
$(NH_4)_2SO_4$ precipitate (30~60%)	47	21.4
Active Mono Q fractions	875	0

Fig. 2. FAB mass spectrum of pirlimycin inactivation product.

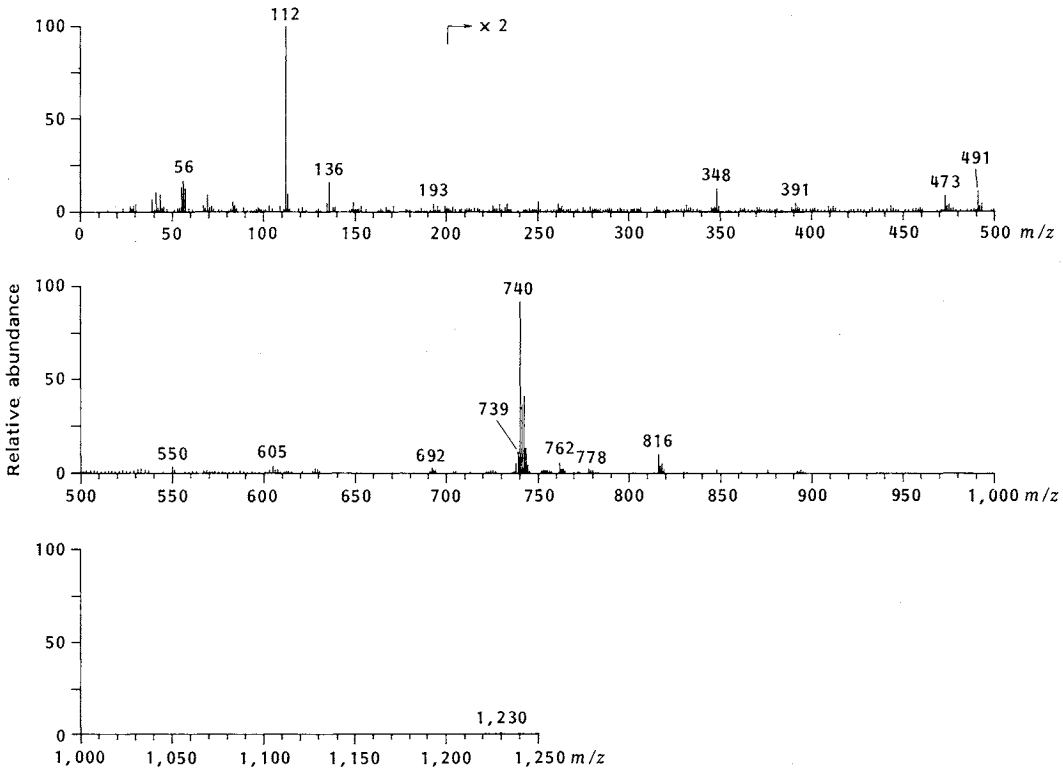


Table 2 presents data showing that the *S. coelicolor* Müller cell-free extract and the $(NH_4)_2SO_4$ precipitate catalyzed pirlimycin and oleandomycin inactivation by the two reactions indicated. However, the pooled fractions from a Mono Q anion exchange column are able only to catalyze pirlimycin ribonucleotidylation. Data presented here indicate the lincosaminide *O*-nucleotidyltransferase to be separated and distinct from the macrolide-2′-phosphotransferase also present in the *S. coelicolor* Müller cell-free extract.

The pirlimycin conversion products formed using the 35-fold purified enzymes in the presence of ATP and Mg^{2+} at a reaction pH of 6.3 were determined to be pirlimycin-3-(5′-adenylate) and possibly pirlimycin-3-*O*-phosphate.

The FAB mass spectrum of the pirlimycin conversion product is shown in Fig. 2. The pseudomolecular ion $(M+H)^+$ appears at *m/z* 740. This corresponds to the MW of the pirlimycin-3-(5′-adenylate) ion (Table 3). The ions above this pseudomolecular ion, which appear at *m/z* 762 and 816 are due to adducts of Na

Table 3. FAB-MS data for pirlimycin and pirlimycin inactivation products.

Compound	Formula	MW	Observed ion $(M+H)^+$
Pirlimycin	$C_{17}H_{31}Cl_1N_2O_5S_1$	410	411
Pirlimycin-3-*O*-phosphate	$C_{17}H_{32}Cl_1N_2O_8P_1S_1$	490	491
Pirlimycin-3-(5′-adenylate)	$C_{27}H_{43}Cl_1N_7O_{11}P_1S_1$	739	740

Table 4. ^1H and ^{13}C NMR spectra of pirlimycin and pirlimycin-3-(5′-adenylate).

Atom No.	δ_H (ppm)		δ_C (ppm)		Atom No.	δ_H (ppm)		δ_C (ppm)	
	Pirlimycin	Pirlimycin adenylate	Pirlimycin	Pirlimycin adenylate		Pirlimycin	Pirlimycin adenylate	Pirlimycin	Pirlimycin adenylate
1	5.18, 5.20	5.28	87.8 (d)	86.4 (d)	14_β	2.86	2.79	42.8 (t)	43.0 (t)
S-CH_3	2.04	2.04	12.2 (q)	12.0 (q)	14_α	3.22	3.16	—	—
2	3.92	4.07	67.5 (d)	66.8 (d)	15	1.26	1.20	28.2 (t)	28.2 (t)
3	3.38	3.88	70.1 (d)	74.4 (d)	16	0.89	0.83	10.8 (q)	10.7 (q)
4	3.82	4.03	67.2 (d)	67.0 (d)	1′	—	5.96	—	87.4 (d)
5	4.10	4.05	69.0 (d)	69.0 (d)	2′	—	4.75	—	70.7 (d)
6	4.39	4.47	52.2 (d)	52.2 (d)	3′	—	4.21	—	73.5 (d)
7	4.50	4.50	59.3 (d)	59.3 (d)	4′	—	4.42	—	83.9 (d)
8	1.34	1.34	22.6 (q)	22.5 (q)	5′	—	4.11	—	65.0 (t)
9	—	—	168.8 (s)	169.3 (s)	2″	—	8.55	—	152.6 (d)
10	3.78	3.94	57.1 (d)	57.2 (d)	4″	—	—	—	149.8 (s)
11_β	1.22	1.16	33.0 (t)	33.0 (t)	5″	—	—	—	118.5 (s)
11_α	2.12	2.08	—	—	6″	—	—	—	155.8 (s)
12	1.51	1.64	35.0 (d)	35.0 (d)	8″	—	8.16	—	139.3 (d)
13_β	1.28	1.41	27.2 (t)	27.6 (t)	6a N-H	8.47	—	—	—
13_α	1.78	1.99	—	—					

(739+23) and matrix (739+77), respectively. The fragment ions at *m/z* 112 and 136 indicate the presence of 4-ethyl-piperidine and adenosine. Finally, the ion at *m/z* 491 could be due to a fragment ion of pirlimycin-3-(5′-adenylate), or could be interpreted as the pseudomolecular ion of pirlimycin-3-*O*-phosphate. It is not possible to exclude the presence of a small amount of pirlimycin phosphate as an impurity in these preparations on mass spectrometric arguments alone. The FAB mass spectrum shown in Fig. 2 was compared with a sample of pure pirlimycin-3-(5′-adenylate)[11] and they were very similar.

Since the FAB-MS data show that the reaction mixture may contain both the adenylate and phosphate inactivated products, 1D and 2D NMR methods were used to characterize the inactivated materials.

The ^1H and ^{13}C NMR data indicated that the sample contained a single component (Fig. 1 and Table 4). The presence of an adenylate moiety is suggested by the ^{13}C NMR signals at 119.5, 139.7, 149.2, 153.1, 155.7 ppm corresponding to C-5″, C-8″, C-4″, C-2″, and C-6″, respectively.[12] The linkage of C-3 of pirlimycin and C-5″ of the adenine moieties by the phosphodiester bonds is suggested by the ~4 ppm downfield shift of the C-3 carbon compared to the corresponding unphosphorylated moieties.[12] Furthermore, the characteristic scalar couplings between ^{13}C and ^{31}P nuclei are also observed for the C-3 ($J=5.6$ Hz), C-4 ($J=1$ Hz), C-2 ($J=5.6$ Hz), C-5′ ($J=5.6$ Hz), and C-4′ ($J=9.0$ Hz). The NMR data, therefore, unambiguously identified the bioconverted product as pirlimycin-3-(5′-adenylate), through the excellent agreement of the ^{13}C NMR chemical shift values of C-1′ through C-5″ to those reported in literature.[12]

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