ENZYMIC INACTIVATION OF LINCOSAMINIDE AND MACROLIDE ANTIBIOTICS: DIVALENT METAL CATION AND COENZYME SPECIFICITIES

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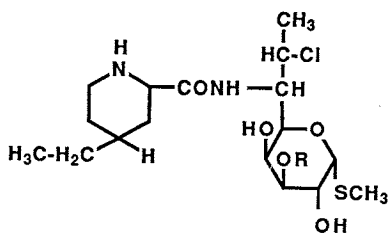
Streptomyces coelicolor Müller, NRRL 3532 (UC 5240) in fermentation converts lincosaminide antibiotics to mixtures of inactive lincosaminide-3-(5′-ribonucleotides) and lincosaminide-3-*O*-phosphates.[1,2] As might be expected, crude enzyme preparations of *S. coelicolor* Müller catalyze the formation of both types of these inactivation products. However, these conversions are pH dependent with nucleotidylation (Fig. 1) occurring optimally near pH 6.0[3,4] and phosphorylation (Fig. 1) near pH 8.5.[5] The *S. coelicolor* Müller crude enzyme also catalyzes the inactivation of erythromycin, oleandomycin, spiramycin, leucomycin A_3 and tylosin (Fig. 2) through phosphorylation at their 2′-positions,[6] while *S. coelicolor* Müller in fermentation has been shown to similarly convert oleandomycin and spiramycin.[7] By manipulation of reaction pH and substrate, the crude enzyme can be employed in the investigation of macrolide phosphorylation, lincosaminide phosphorylation and lincosaminide nucleotidylation.

In these currently reported investigations of the crude enzyme, pirlimycin (Fig. 1) and tylosin (Fig. 2) were employed respectively as the lincosaminide and macrolide test substrates. We have reported the coenzyme and metal cofactor specificities, as well as the optimal times of production of the conversion enzyme activities by *S. coelicolor* Müller.

Growth of *S. coelicolor* and Preparation of the Crude Enzyme

S. coelicolor Müller was grown as described in two of our previous publications,[3,6] and was harvested by centrifugation in the cold at $10^4 \times g$ for 15 minutes. Sedimented mycelial mass (10 g) was resuspended in 10 ml of 100 mM potassium phosphate (pH 7.0) and was centrifuged as described previously. The washed cellular material was then resuspended in 10 ml of 10 mM potassium phosphate (pH 7.5), which contained EDTA at 500 μg/ml and was lysed by egg-white lysozyme (Sigma) (2 mg/ml of resuspended mycelia) using the procedure of HEY and ELBEIN.[8] The resulting cell free ex-

Fig. 1. The structures of lincosaminide substrate and products.



Pirlimycin R=H

Pirlimycin-3-phosphate R=$\overset{O}{\overset{\|}{P}}(OH)_2$

Pirlimycin-3-(5′-adenylate) R=

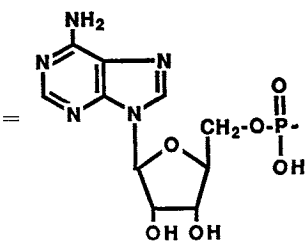
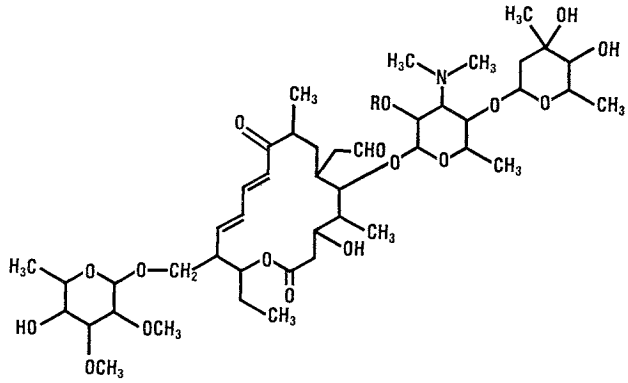


Fig. 2. The structure of tylosin and tylosin-2′-*O*-phosphate.



Tylosin R=H

Tylosin-2′-*O*-phosphate R=$\overset{O}{\overset{\|}{P}}(OH)_2$

tract was used as the crude enzyme. Crude enzyme protein was quantitated by the Bio-Rad method which is based on the procedure of BRADFORD.[9] The protein concentration of the crude enzyme averaged *ca.* 4 mg/ml. In all cases, phosphate buffers were prepared by adjusting the pH of solutions of KH_2PO_4 with KOH.

Enzymic Nucleotidylation of Pirlimycin

This procedure was performed using reaction volumes of 20 ml. Twenty-five-ml Erlenmeyer flasks were employed as the reaction vessels. The reaction mixtures contained nucleoside-5′-triphosphates (pH 7.0) (Sigma) 50 μmol, crude enzyme protein 400 μg, divalent metal cations 40 μmol, pirlimycin·HCl (Upjohn) 223 μg and potassium phosphate (pH 7.0) 5 μmol added per ml of distilled water. The reaction mixtures were adjusted to pH 6.0 with HCl and the reactions were stirred with small magnetic bars at 25°C for the designated periods of time.

Using the reaction conditions described, pirlimycin and other lincosaminides are known to be inactivated through conversion to lincosaminide-3-(5′-ribonucleotides).[3,4] Therefore, inactivation under these conditions is known to be equivalent to nucleotidylation. A biounit 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 Schuell No. 740-E) produces a zone of growth inhibition of 20 mm when applied to seeded agar trays. Through comparison of the experimental biounit activities to those produced by standardized pirlimycin solutions, μg values were assessed. Recently, crude enzyme preparations of Staphyloccocci were reported to convert lincomycin and clindamycin to their ribonucleotides in the presence of Mg^{2+} and ATP, GTP, UTP or CTP.[10]

Enzymic Phosphorylation of Pirlimycin

The reaction conditions were identical to those reported above for pirlimycin nucleotidylation with the following exceptions. Tris buffer (pH 8.0) was added at a concentration of 5 μmol/ml of reaction volume and the reaction pH was adjusted to pH 8.5 with KOH. Under these reaction conditions, lincosaminides are known to be converted to lincosaminide-3-*O*-phosphates.[5] Pirlimycin quantitation was performed as described above. Tris buffer was prepared by adjusting the pH of solutions of Tris base with HCl.

Enzymic Phosphorylation of Tylosin

Enzymic phosphorylation of tylosin was performed using a reaction volume of 20 ml. Twenty-five-ml Erlenmeyer flasks were employed as the reaction vessels. The reaction mixtures contained nucleoside-5′-triphosphates (pH 7.0) (Sigma) 50 μmol, tylosin tartrate (Sigma) 550 μg, crude enzyme protein 400 μg, divalent metal cations 40 μmol and potassium phosphate (pH 6.5) 5 μmol added per ml of distilled water.

These mixtures were adjusted to pH 7.0 with KOH, and the reactions were stirred slowly for the designated periods of time at 25°C. Using these reaction conditions, tylosin is known to be inactivated through conversion to tylosin-2′-*O*-phosphate.[6] Quantitation of tylosin was performed as described for pirlimycin.

Divalent Metal Cation Specificity

Previous publications from our laboratories have reported a requirement for Mg^{2+} in the inactivation of lincosaminide[3~5] and macrolide[6] antibiotics as catalyzed by crude enzyme preparations of *S. coelicolor* Müller. As part of the current study we have investigated the specificities of divalent metal cations in these conversions of pirlimycin and tylosin. As seen in Table 1, tylosin was phosphorylated in the presence of Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} or Mn^{2+}. Pirlimycin was phosphorylated in the presence of Mg^{2+}, Ca^{2+} or Co^{2+}, and nucleotidylated in the presence of Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} or Mn^{2+}. These differences in cation specificities suggest differences in the enzymes catalyzing lincosaminide phosphorylation and nucleotidylation.

Coenzyme Specificity

Previous publications have also shown requirements for various nucleoside-5′-triphosphates in the nucleotidylation of pirlimycin,[3,4] the phosphorylation of tylosin,[6] and in the case of the phosphorylation of lincosaminides.[5] Data presented in Table 2 compare the coenzyme specificities of all three conversions. Tylosin was phosphorylated effectively in the presence of ATP or UTP, and to a lesser extent in the presence of GTP, while pirlimycin was phosphorylated and nucleotidylated in the presence of ATP, UTP, GTP, CTP or ITP. These coenzyme specificities indicate differences in the lincosaminide and macrolide conversion enzymes.

Crude Enzyme Specific Activity vs. Microbial Age at Harvest

The specific activities related to the three conversions catalyzed by the *S. coelicolor* Müller crude enzyme were investigated as a function of the age of the mycelia at harvest. Data presented in Table 3 indicate 24 hours to be the optimal age of *S. coelicolor* Müller for use in crude enzyme preparation. The lack of significant change in crude enzyme specific activity between 24 and 72 hours in the case of the phosphorylation of tylosin would also indicate differences in the enzymes catalyzing pirlimycin and tylosin conversions. Note the significant change in specific activity during the same time

Table 1. Divalent metal cation specificity for antibiotic inactivation.

Antibiotic	Reaction	Metal cation	Antibiotic remaining (nmol/ml)				
			0.5 hour	4 hours	11 hours	23 hours	49 hours
Tylosin	Phosphorylation	Mg^{2+}	587	470	335	205	190
		Ca^{2+}	587	470	335	205	190
		Co^{2+}	587	470	469	294	235
		Zn^{2+}	587	470	469	294	235
		Mn^{2+}	587	470	335	294	190
		None	587	587	568	469	411
Pirlimycin	Phosphorylation	Mg^{2+}	394	—	49	0	0
		Ca^{2+}	394	—	101	0	0
		Co^{2+}	398	—	233	119	16
		Zn^{2+}	398	—	374	280	350
		Mn^{2+}	398	—	374	280	350
		None	394	—	398	374	403
Pirlimycin	Nucleotidylation	Mg^{2+}	362	0	0	0	—
		Ca^{2+}	362	329	262	72	—
		Co^{2+}	362	31	31	13	—
		Zn^{2+}	362	16	16	11	—
		Mn^{2+}	362	329	83	0	—
		None	362	329	329	277	—

Table 2. Coenzyme specificity for antibiotic inactivation.

Antibiotic	Reaction	Coenzyme	Antibiotic remaining (nmol/ml)				
			0.5 hour	4 hours	11 hours	23 hours	49 hours
Tylosin	Phosphorylation	ATP	585	548	205	146	41
		UTP	585	548	293	190	59
		GTP	585	548	410	296	235
		CTP	577	552	431	410	330
		ITP	577	552	431	410	330
		None	577	552	504	469	469
Pirlimycin	Phosphorylation	ATP	559	280	56	0	—
		UTP	559	394	197	65	—
		GTP	559	454	183	0	—
		CTP	559	454	56	0	—
		ITP	559	454	47	0	—
		None	559	559	559	400	—
Pirlimycin	Nucleotidylation	ATP	559	0	0	0	—
		UTP	559	215	0	0	—
		GTP	559	27	0	0	—
		CTP	559	141	0	0	—
		ITP	559	0	0	0	—
		None	559	559	559	559	—

Table 3. Crude enzyme specific activity vs. microbial age at harvest.

Harvest (hours)	Substrate	Reaction	Specific activity (nmol/hour/mg protein)
24	Pirlimycin	Nucleotidylation	233
	Pirlimycin	Phosphorylation	66
	Tylosin	Physphorylation	37
48	Pirlimycin	Nucleotidylation	18
	Pirlimycin	Phosphorylation	20
	Tylosin	Phosphorylation	25
72	Pirlimycin	Nucleotidylation	3
	Pirlimycin	Phosphorylation	32
	Tylosin	Phosphorylation	36

period concerning the nucleotidylation of pirlimycin and the smaller, but significant change concerning the phosphorylation of pirlimycin between 24 and 48 hours (Table 3).

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