Journal of Industrial Microbiology, 1 (1986) 17–21
Elsevier

SIM 004

Enzymic nucleotidylylation of lincosaminide antibiotics

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Received 15 July 1985
Revised manuscript received 11 December 1985
Accepted 11 December 1985

Key words: Lincosaminides; Lincomycin; Pirlimycin; Clindamycin; Inactivation; Nucleotidylylation; *Streptomyces*

SUMMARY

Fermentations of *Streptomyces coelicolor* are known to convert lincosaminide antibiotics to mixtures of their inactive 3-(5′-ribonucleotides). In the present study, lincomycin, clindamycin and pirlimycin were nucleotidylylated and inactivated using crude enzyme preparations of *S. coelicolor*. Optimal conversion is known to occur near pH 6 and to require Mg^{2+} and nucleoside 5′-triphosphates. In descending order of activity, inosine, adenosine, guanosine, cytidine and uridine 5′-triphosphates functioned as cofactors in these nucleotidylylations. In all instances, 90% of maximal conversion occurred within 24 h. When reaction rates were investigated as functions of enzyme protein addition, pirlimycin appeared to be the superior lincosaminide substrate. Antibiotic activities of these inactivation products could be regenerated through the action of phosphodiesterase, EC 3.1.4.1.

INTRODUCTION

Cultures of *Streptomyces coelicolor* have been reported to convert lincosaminide antibiotics (Fig. 1) to mixtures of their inactive 3-(5′-ribonucleotides) and 3-phosphates [1,2]. As might be expected, cell-free preparations of *S. coelicolor* in the presence of nucleoside 5′-triphosphates catalyze the formation of both types of these inactivation products. However, these conversions are pH dependent with ribonucleotidylylation (Fig. 2) occurring optimally near pH 6 [6]. When such cell-free conversion was attempted at pH 8, the lincosaminide substrates were converted to their 3-phosphates [2,4]. Therefore, these mechanisms of enzymic inactivation of lincosaminide antibiotics appear to be dependent on reaction pH.

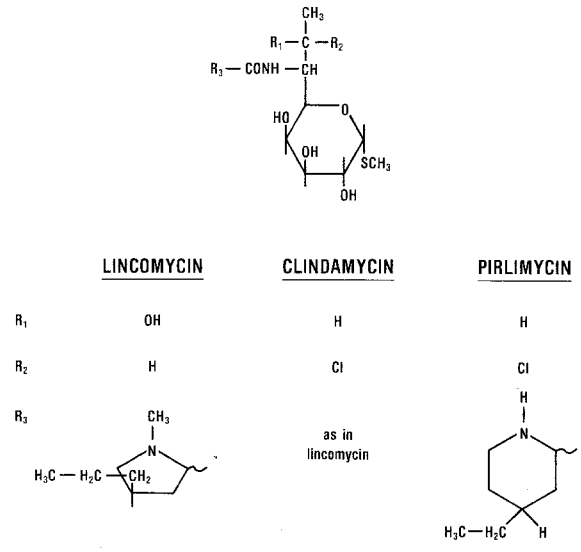

Fig. 1. Structures of lincomycin, clindamycin and pirlimycin.

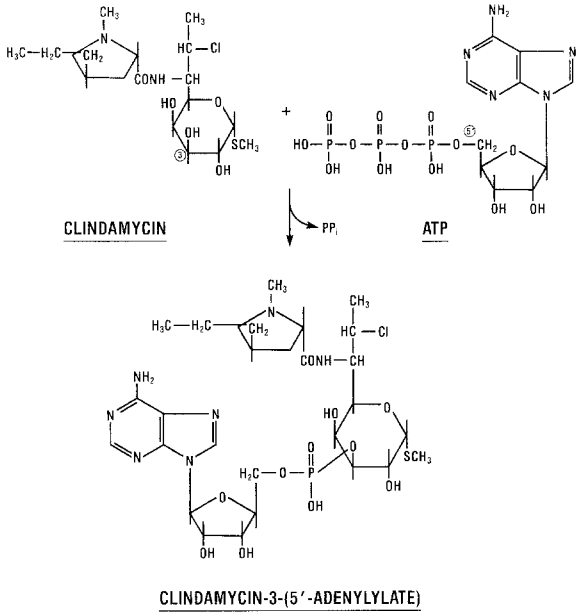

Fig. 2. Adenylylation of clindamycin.

METHODS

Fermentation conditions

S. coelicolor, UC® 5240 (NRRL 3532), was stored and maintained on sterile soils in the culture collection of The Upjohn Company. The organism was inoculated into a seed medium (GS-7) which contained Cerelose (C.P.C. International) and Pharmamedia (Proctor and Gamble), each added at 25 g per l of tap H_2O. The medium was adjusted to pH 7.2 with NH_4OH and was autoclaved for 30 min. The inoculated 100 ml volumes of GS-7 were shaken in wide-mouth 500 ml fermentation flasks at 250 rpm for 48 h at 28°C. The mature seed cultures were used as the source of inoculum (5% seed rate) for the fermentation medium. The latter was a modification of a medium reported by Coats and Argoudelis [4] and contained glucose 20 g, NZ-amine B (Sheffield Chemicals) 5 g, yeast extract (Difco) 2.5 g, $NaNO_3$ 1.5 g, and $FeSO_4$ 10 mg added per l of deionized H_2O. After formulation, the medium was adjusted to pH 7.2 with NH_4OH and was sterilized by autoclaving. The medium was employed in the manner described for GS-7. After 24 h of growth at 28°C the fermentation was centrifuged in the cold at $10^4 \times g$ for 15 min. The sedimented cellular mass was retained as the source of enzyme.

Preparation of the cell-free extract (CFX)

Sedimented cellular mass (100 g) was resuspended in 100 ml of 100 mM potassium phosphate (pH 7) and was centrifuged as described previously. The washed cellular material was then resuspended in 100 ml of 10 mM potassium phoshpate (pH 7.5), which contained EDTA at 500 mg per liter and was lysed by egg-white lysozyme (Sigma, grade I) using the procedure of Hey and Elbein [5]. The resulting CFX was used as the crude enzyme. Crude enzyme protein was quantitated by the Bio-Rad method which is based on the procedure of Bradford [3]. The protein concentrations of the crude enzymes averaged 5 mg per ml.

Enzymic nucleotidylylation of lincosaminides

This procedure was performed using reaction volumes ranging between 5 ml and 2 l. The reaction mixtures contained nucleotide 5'-triphosphates (Sigma Chemicals) 50 μmol, lincosaminide antibiotics (The Upjohn Company) 0.2–2 μmol, crude enzyme protein 100–1000 μg, $MgCl_2$ 40 μmol and potassium phosphate 5 μmol per ml of distilled H_2O. This mixture was adjusted to pH 6 and the reactions were stirred at 25°C for the designated period of time.

Isolation and characterization of lincosaminide-3-(5'-ribonucleotides)

Isolation and characterization of the reaction products synthesized using the previously described biochemical procedure were performed as described in U.S. Patent 4,430,495 [6].

Inactivation of lincosaminide antibiotics

Using the reaction conditions described, lincosaminide antibiotics (clindamycin, pirlimycin and lincomycin) were inactivated through conversion to their 3-(5'-ribonucleotides) [6]. As these inactivations at pH 6 led almost exclusively to the formation of lincosaminide 3-(5'-ribonucleotides), inactivation under these conditions was considered to

be equivalent to nucleotidylylation. A biounit assay employing *Micrococcus luteus,* UC® 130, was used to routinely 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), produced a zone of growth inhibition of 20 mm when applied to a seeded agar culture. Using this assay, 1 biounit was equivalent to 1 μg of clindamycin, 2 μg of pirlimycin or 3 μg of lincomycin.

Enzymic hydrolysis of lincosaminide-3-(5'-ribonucleotides)

Hydrolysis of lincosaminide-3-(5'-ribonucleotides) catalyzed by phosphodiesterase, EC 3.1.4.1 (Sigma Chemicals), was performed as described previously [1,2,6]. Analysis was performed using a biological assay similar to that described above with zone size indicating activity.

RESULTS AND DISCUSSION

Table 1 shows the biochemical requirements for enzymic nucleotidylylation of lincosaminide antibiotics as catalyzed by a crude enzyme preparation of *S. coelicolor.* Data concerning the complete reaction mixture demonstrate the total inactivation of these antibiotics to occur within 24 h. However, in the absence of CFX, ATP or Mg^{2+} only minimal inactivation occurred. These data show the reaction to be enzyme catalyzed and to require ATP and Mg^{2+}.

Table 1

Enzyme and cofactor requirements for inactivation of lincosaminides

Reaction conditions	Assay time (h)	Lincomycin (μg per ml)	Pirlimycin (μg per ml)	Clindamycin (μg per ml)
Complete	0.25	249	308	307
	5	81	64	90
	24	0	0	0
Minus ATP	0.25	270	298	307
	5	249	298	307
	24	231	256	281
Minus Mg^{2+}	0.25	270	298	338
	5	249	298	333
	24	231	256	281
Minus CFX	0.25	249	298	307
	5	231	298	307
	24	210	230	256

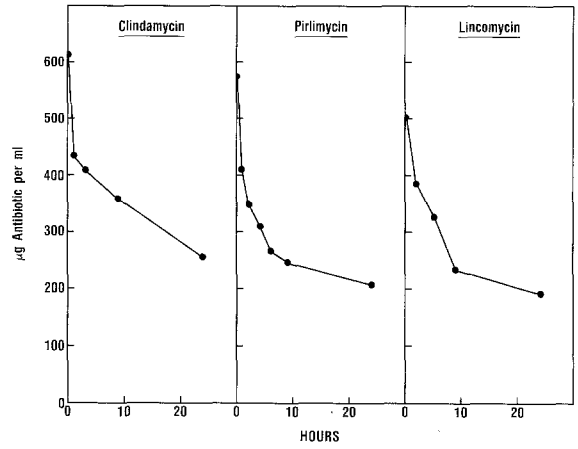

Fig. 3. Rates of enzyme catalyzed inactivation of lincosaminide antibiotics.

The time course of this reaction as applied to the adenylylation of clindamycin, pirlimycin and lincomycin is shown in Fig. 3. As the lincosaminide

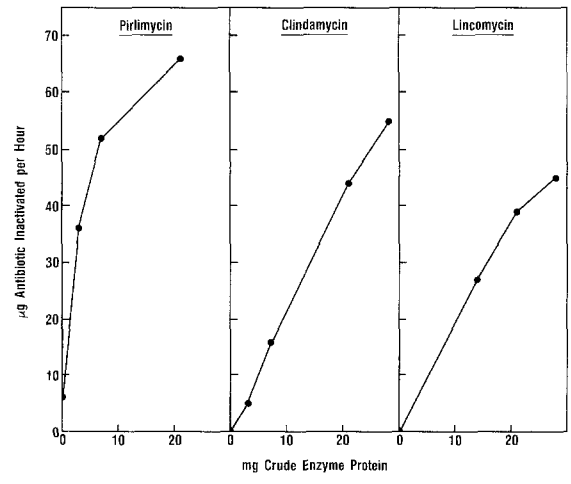

Fig. 4. Crude enzyme specific activity.

Table 2

Specificity of nucleoside triphosphate-linked inactivation of lincosaminides

Nucleoside triphosphate employed	Assay time (h)	Lincomycin (μg per ml)	Pirlimycin (μg per ml)	Clindamycin (μg per ml)
ITP	0.25	196	192	184
	4	58	29	13
ATP	0.25	196	249	174
	4	58	68	43
GTP	0.25	196	192	164
	4	97	48	43
CTP	0.25	196	249	184
	4	97	135	102
UTP	0.25	173	231	174
	4	115	162	102
None	0.25	196	249	184
	4	184	249	164

substrates were present at higher concentrations than employed in the previous experiment (Table 1), their total inactivation did not occur in 24 h. The higher lincosaminide substrate levels were employed so that their concentrations would not become limiting during the 24 h reaction period. Note that after 5 h of incubation, pirlimycin, lincomycin and clindamycin were present at 50, 60 and 80% of their respective initial concentrations. This finding indicates that pirlimycin is the superior substrate in this enzymic adenylylation of lincosaminide antibiotics.

Fig. 4 shows the rates of lincosaminide adenylylation investigated as functions of crude enzyme protein addition. The reaction rates employed in these analyses were those obtained in the initial 150 min of inactivations similar to those presented in Fig. 3. Data presented in Fig. 3, as do those in Fig. 4, indicate pirlimycin to be the superior lincosaminide substrate tested.

Table 2 shows the specificity of the crude enzyme for nucleoside 5′-triphosphates. These data indicate the purine-derived 5′-triphosphates to be superior to their pyrimidine-derived counterparts. The nucleoside 5′-triphosphates which appear in Table 2 are listed roughly in order of their abilities to serve in these nucleotidylylations.

The antimicrobial activities of the inactive lincosaminide 3-(5′-ribonucleotides) are known to be regenerated through the action of phosphodiesterase, EC 3.1.4.1 [1,6]. Data presented in Table 3 show the ability of this enzyme to regenerate clindamycin and pirlimycin activities from their adenylylates. Such regeneration suggests the abilities of lincosaminide 3-(5′-ribonucleotides) to serve as antibiotic prodrugs.

Table 3

Reactivation of lincosaminide ribonucleotides by phosphodiesterase (EC 3.1.4.1).

Substrate	Enzyme	*M. luteus* growth inhibition (mm zone)		
		0.25 h	2 h	18 h
Clindamycin	+	23	37	44
Adenylylate	−	0	0	0
Pirlimycin	+	22	34	41
Adenylylate	−	0	0	0

ACKNOWLEDGEMENT

Thanks are extended to Nancy K. Hopkins and Alice H. Lin for protein quantitation.

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