ENZYMATIC ADENYLYLATION OF SPECTINOMYCIN BY *ACINETOBACTER CALCOACETICUS* SUBSP. *ANITRATUS*

SHOJI SHIMIZU, MATSUHISA INOUE and SUSUMU MITSUHASHI

Department of Microbiology, School of Medicine, Gunma University, Maebashi, Japan

HIROSHI NAGANAWA and SHINICHI KONDO

Institute of Microbial Chemistry, Shinagawa, Tokyo, Japan

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Spectinomycin (SPC) was inactivated in the presence of adenosine-5′-triphosphate and magnesium ion by an enzyme preparation made from *Acinetobacter calcoaceticus* subsp. *anitratus* GN12313 resistant to both SPC and streptomycin. The structure of the inactivated SPC was found to be the adenylylated product of the hydroxy group on C-9 of the actinamine moiety.

It has been reported that spectinomycin (SPC) is inactivated by cell-free extracts of SPC-resistant strains of *Escherichia coli*[1,2], *Staphylococcus aureus*[3] and *Acinetobacter calcoaceticus* subsp. *anitratus*[4] in the presence of adenosine-5′-triphosphate (ATP) by adenylylation of the drug. There are two known adenylyltransferases (AAD) for SPC. The streptomycin (SM)- and SPC-adenylyltransferase adenylylates both SM and SPC[1] but the SPC-adenylyltransferase inactivates SPC but not SM[3]. The chemical structure of adenylylated SPC has not conclusively established. Recently we have isolated SM·SPC-resistant strains of *A. calcoaceticus* subsp. *anitratus* from clinical materials. This paper deals with their inactivation mechanism and the chemical structure of the inactivated SPC.

Materials and Methods

Bacterial Strain

Acinetobacter calcoaceticus subsp. *anitratus* GN12312, GN12313, GN12314, and GN12315 were isolated from clinical specimens. *E. coli* K-12 ML4905 (*met. rif*^{r}) (*rif*^{r}, resistant to rifampin) and *E. coli* K-12 ML1410 (*met, nal*^{r}) (*nal*^{r}, resistant to nalidixic acid) were used as the recipients for the attempted transfer of drug resistance.

Antibiotics and Chemicals

The following drugs were used; tetracycline (TC), chloramphenicol (CM), streptomycin (SM), sulfanilamide (SA), kanamycin (KM), carbenicillin (CBPC), ampicillin (APC), rifampin (RIF), and nalidixic acid (NA). Spectinomycin (SPC) was supplied by the Upjohn Co., Kalamazoo, U.S.A. Disodium ATP was purchased from the Kyowa Hakko Co., Tokyo. Phosphodiesterase was purchased from the Sigma Chemical Co. The isotope-labeled preparations of ATP, *i.e.*, [γ-^{32}P]ATP (3 Ci/m mole) and [8-^{14}C]ATP (58 mCi/m mole), were purchased from the Radiochemical Centre, Amersham, England.

Determination of Drug Resistance

One loopful (about 5 μl) of 100-fold diluted overnight culture dilution (about 10^8 cells/ml) of each strain was spotted on heart infusion (HI) agar plates containing serial twofold dilutions of each antibiotic. The minimal inhibitory concentration of each drug was scored after 18 hours of incubation at 37°C.

Media

Medium B was used for liquid culture and consisted of Na_2HPO_4 (7.0 g), KH_2PO_4 (2.0 g), $(NH_4)_2SO_4$ (1.2 g), $MgSO_4 \cdot 7H_2O$ (0.4 g), glucose (2.0 g), peptone (Daigo, Osaka, 10 g), yeast extract (Difco, 1.0 g), and 1,000 ml of deionized water (pH 7.2). For the determination of drug resistance, heart infusion (HI) agar (Eiken, Tokyo) and peptone water were used. Peptone water consisted of 1,000 ml of distilled water, 5 g of NaCl, and 10 g of peptone. For the assay of SA resistance, a semisynthetic medium was used consisting of 1,000 ml of medium A (5), 2 g of casamino acids (Difco), 10 mg of tryptophan, 1 mg of nicotinic acid, 10 mg of thiamine·HCl, and 2 g of glucose. Penassay broth (Difco) was used for the test of elimination of drug resistance.

Inactivation of Antibiotic by Cell-free Extracts

The reaction mixture consisted of 150 μl of the S-105 fraction (36 mg of protein per ml), 50 μl of 1 mM antibiotic, 50 μl of 20 mM ATP, 100 μl of 20 mM magnesium acetate, 50 μl of 0.5 M acetate buffer (pH 6.0) and 100 μl of distilled water and was incubated at 37°C for 6 hours. After heating at 100°C for one minute, the residual activity of drug in the reaction mixture was determined by using *B. subtilis* ATCC6633 as a test organism. The incorporation of isotope from labeled ATP into drug was investigated by the method of OZANNE *et al.*[6]. The reaction mixture contained 80 μl of the S-105 fraction (1.2 mg of protein per ml), 10 nmole of [8-^{14}C]ATP (58 mCi/mmole) or [γ-^{32}P]ATP (3 Ci/mmole), 20 nmole of each drug, 24 μmole acetate buffer (pH 6.0), and 800 n mole of magnesium acetate, in a total volume of 100 μl. Experimental procedure was as described previously[3].

Isolation of Enzymatically Inactivated Spectinomycin

The enzymatic inactivation of SPC was carried out at 37°C for 6 hours in the following reaction mixture, *i.e.*, 178 ml of the S-105 fraction (36 mg of protein per ml), 5.06 g of disodium ATP, 6.86 g of magnesium acetate, 200 mg of SPC, and 69 ml of 0.5 M acetate buffer (pH 6.0). The total volume of the reaction mixture was brought to 627 ml with distilled water and incubated at 37°C. After 6 hours of incubation, the reaction was stopped by heating in boiling water for 8 minutes. The supernatant obtained by centrifugation at 6,000$\times g$ for 30 minutes was passed through a column of Amberlite CG-50 (H^+ form, 133 ml). The column was washed with 800 ml of distilled water and the inactivated SPC eluted with 0.5 N HCl. The eluate giving a positive anthrone reaction and showing no antibacterial activity was collected. The eluate was subjected to rechromatography on Amberlite CG-50 (H^+ form, 40 ml) and the inactivated SPC was eluted with 0.01 N HCl. The fraction giving a positive anthrone reaction and showing no antibacterial activity was neutralized with NaOH, concentrated under vacuum and the concentrated eluate passed through a column of Sephadex G-10 (260 ml). The fraction giving a positive anthrone reaction and showing no antibacterial activity was again concentrated under vacuum and the inactivated SPC precipitated by addition of acetone and dried *in vacuo*; 90 mg of inactivated SPC was obtained.

Elimination of Drug Resistance

Five μl of overnight broth culture of a test strain was inoculated in 10 ml of fresh broth containing various concentrations of ethidium bromide. After incubation at 37°C for 18 hours, the culture treated with the highest concentration of drug showing visible growth was selected, and an appropriate dilution was spread on an agar plate. After 18 hours of incubation at 37°C, the loss of resistance in each colony was tested by replica-plating.

Conjugal Transfer of Resistance

Two ml of recipient and 0.5 ml of donor culture were mixed in early stationary phase of growth and the mixture filtered through a 0.45 μ Millipore filter. The filter was incubated for 2 hours at 37°C on the surface of a HI plate and then vigorously whirlimixed for one minute with 5 ml of Penassay broth to resuspend the cells. The resulting suspension was then plated on the appropriate selective media.

Treatment of Inactivated Spectinomycin with Phosphodiesterase

The following reaction mixture was used: 50 μg of inactivated SPC, 10 μg of phosphodiesterase (venom phosphodiesterase, Sigma Chemical Company) and 100 μl of GMK solution (0.05 M KCl and

0.01 M $MgCl_2$ in 0.05 M glycine-NaOH buffer, pH 9.0) in a total volume adjusted with distilled water to 200 μl. The reaction was performed at 37°C for 17 hours. The reactivation of the inactivated SPC in the solution was determined by a paper disk method.

Results

Detection of SM·SPC-adenylylating Enzyme

Four strains of *A. calcoaceticus* subsp. *anitratus* resistant to both SM and SPC were selected from our stock cultures. The minimal inhibitory concentrations (MIC) of several drugs toward these strains are shown in Table 1. GN12313 was susceptible to TC, CM, SA, APC and KM but highly resistant to both SM and SPC. The mechanism of inactivation of both SM and SPC was studied and as shown in Table 2, the incorporation of ^{14}C from [8-^{14}C]ATP into both SM and SPC occurred in the presence of S-105 extracts of the four strains. Incorporation of ^{32}P from [γ-^{32}P]ATP could not be demonstrated, indicating that the SM·SPC-resistant strains inactivated both SM and SPC by adenylylation.

Conjugal Transfer of Resistance

To investigate the genetic properties of the SM·SPC-resistant determinant(s), the transferability of SM·SPC-resistance was examined. The SM·SPC-resistance in four *A. calcoaceticus* subsp. *anitratus* strains could not be transferred to two *E. coli* K12 strains. The SM·SPC-resistance of the four strains could not be cured by treatment with ethidium bromide. Plasmid DNAs of at least three different molecular weights could be demonstrated in GN12313 by agarose gel electrophoresis of covalently closed circular (ccc) DNA band obtained by cesium chloride-ethidium bromide density gradient centrifugation.

Table 1. Minimum inhibitory concentrations of drugs against *Acinetobacter calcoaceticus* subsp. *anitratus*.

Strain	MIC (μg/ml)							
	TC	CM	SM	SPC	SA	KM	APC	Hg
GN12312	1.6	50	100	100	1,600	100	25	6.3
GN12313	1.6	3.2	200	800	12.5	0.4	25	6.3
GN12314	6.3	50	400	400	200	25	50	3.2
GN12315	3.2	50	800	800	25	3.2	50	50

Table 2. Inactivation of SM and SPC by cell-free extracts from SM·SPC-resistant strains of *Acinetobacter calcoaceticus* subsp. *anitratus*.

Strain	MIC[a] (μg/ml)		Inactivation[b]		Incorporation of labeled ATP into drug (counts/minute)			
					^{14}C		γ-^{32}P	
	SM	SPC	SM	SPC	SM	SPC	SM	SPC
GN12312	100	100	+	+	1,058	760	0	0
GN12313	200	800	+	+	56,409	36,118	0	0
GN12314	400	400	+	+	489	423	0	0
GN12315	800	800	+	+	13,038	7,360	0	0

[a] Minimum inhibitory concentration.
[b] See Materials and Methods.

Table 3. Conjugal transfer of SM and SPC resistance in strain GN12313.

Donor	Recipient	Selective drug	Transfer frequency	Resistance pattern of exconjugants
GN12313 (SM·SPC)	ML4905	SM+RIF	$<10^{-8}$	
		SPC+RIF	$<10^{-8}$	
ML4905 (RP4)[a]	GN12313 (SM·SPC)	SM+KM	2.0×10^{-5}	KM, TC, CPC, SM, SPC (8/ 8)
GN12313 (RP4. SM·SPC)[b]	ML4905	KM+RIF	0.8	KM, TC, CPC (150/150)
		SM+RIF	2.0×10^{-7}	KM, TC, CPC, SM, SPC (12/ 12)
ML4905 (RP4. SM·SPC)[c]	ML1410	KM+NA	1.5	KM, TC, CPC, SM, SPC (120/120)
		SM+NA	1.0	KM, TC, CPC, SM, SPC (117/117)

[a] RP4 confers resistance to TC, CM and CPC on its host bacteria.
[b] Exconjugant GN12313 (RP4. SM·SPC) was obtained by the conjugal transmission of RP4 to GN12313 (SM·SPC).
[c] Exconjugant ML4905 (RP4. SM·SPC) was obtained by the conjugal transmission of RP4 to ML4905.

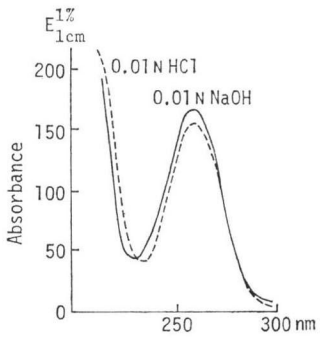
However, when we used *E. coli* K-12 strains as recipients, no transformant which show the ability to produce AAD with plasmid DNAs isolated from GN12313 was isolated. Therefore, the function of these ccc DNA plasmids remains cryptic. We investigated the mobilization of the SM·SPC-resistance determinant in *Acinetobacter* GN12313 in the presence of RP4 to *E. coli* recipient, since other workers have described the introduction an RP4 into *Acinetobacter* and mobilization of the chromosomal markers[7,8], we mated *E. coli* ML4905/RP4 with *Acinetobacter* GN12313 (Table 3). RP4 could be transferred into GN12313 at a frequency of 2.0×10^{-5} per donor cells. Subsequent mating of the transconjugant with an *E. coli* ML4905 recipient showed the transfer of RP4 at a frequency of 0.8 per donor when selected with kanamycin; the transfer frequency of the determinant of AAD activity was 2.0×10^{-7} per donor selected by SM. *E. coli* ML4905 transconjugants obtained in the above experiments were able to transfer RP4 at a high frequency and were capable of producing AAD to *E. coli* ML1410 strain. The transfer frequency of SM·SPC-resistance was almost the same as that of RP4, and all of the transconjugants isolated by either SM or KM selection possessed the resistance markers of RP4 and ability to produce AAD.

These data suggested that a stable recombinant was obtained between RP4 and the determinant of SM·SPC-resistance. It is not known if the gene for AAD was transposed on RP4.

Characteristics of the Inactivated Spectinomycin

SPC was enzymatically inactivated by *Acinetobacter* GN12313 and the inactivated SPC was purified by the procedure described in Materials and Methods. The inactivated SPC thus obtained showed positive anthrone reaction and with HANE's reagent for phosphorus. The inactivated SPC showed one spot in the following thin-layer chromatographic systems: Rf 0.46 with *n*-propanol - pyridine - acetic acid - water (15: 10: 3: 12), Rf 0.45 with ethanol - water - 28% aqueous ammonia (12: 5: 3), Rf 0.41 with

Fig. 1. UV spectra of adenylyl-SPC.



n-propanol - acetic acid - water (1: 1: 1) and Rf 0.11 with *n*-butanol - acetic acid - water (2: 2: 1). Rf values of SPC with these solvents were 0.53, 0.19, 0.25 and 0.48 in the same chromatographic systems, respectively. In high voltage paper electrophoresis using acetic acid - formic acid - water (75: 25: 900) under 3,500 volts for 30 minutes the inactivated SPC moved 11.7 cm toward the cathode and SPC moved 15.3 cm. As shown in Fig. 1, the inactivated SPC showed a maximum absorption at 260 nm in the ultraviolet spectrum, when dissolved in 0.01 N HCl and 0.01 N NaOH and the UV_{max} was similar to that of adenylic acid. Determination of SPC by the anthrone reaction, determination of adenosine by optical density at 260 nm ($E^{1\%}_{1cm}$ 196) and determination of phosphorus by ALLEN's method indicated the presence of SPC, adenosine and phosphorus in the molar ratio of 1: 1: 1 in the inactivated SPC. Analytical data of inactivated SPC were as follows. Analysis: $C_{14}H_{25}O_8N_2 \cdot C_{10}H_{13}O_6N_5P \cdot 9/2H_2O$; calculated: C, 37.90; H, 6.23; N, 12.89; P, 4.07; found: C, 38.79; H, 5.47; N, 11.57; P, 4.54. The reactivation of the inactivated SPC with phosphodiesterase was tested. The inactivated SPC was reactivated by phosphodiesterase and 490 μg of SPC per mg was recovered after 17 hours of treatment with the enzyme. Thus 80% of the inactivated SPC was reactivated.

Table 4. Chemical shift assignments for carbons of adenylyl-SPC.

Atom	δ, ppm		
	AMP[a]	SPC[b]	Adenylyl-SPC[c]
2′	153.3		151.1
4′	149.1		149.3
5′	118.8		119.3
6′	155.6		154.4
8′	140.4		141.4
1″	87.9		88.6
2″	75.4		75.2
3″	71.4		71.0
4″	85.1[d]		84.3[d]
5″	64.4[e]		66.5[e]
2		69.2	69.1
3		42.3	42.3
4		92.6	92.6
4a		94.4	94.2
5a		66.5	66.1
6		59.5	59.2
7		60.7	60.7
8		62.5	62.6[d]
9		66.9	71.9[d]
9a		70.7	69.4[d]
10a		94.4	94.2
2-CH_3		20.5	20.4
6-N-CH_3		31.3	31.1
8-N-CH_3		31.8	32.1

[a] Data taken from reference 10.
[b] Data taken from reference 9.
[c] Chemical shifts were measured in D_2O in parts per million relative to internal Me_4Si.
[d] Doublet.
[e] Poorly resolved doublet.

Structure of Enzymatically Adenylylated Spectinomycin

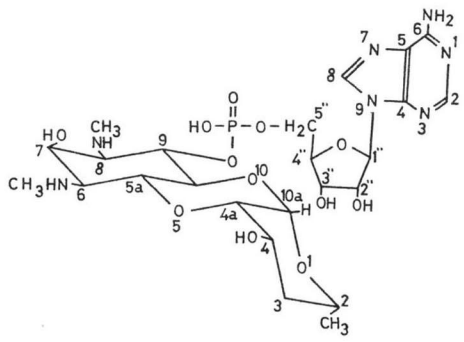
We examined the ^{13}C-magnetic resonance (cmr) spectrum of the inactivated SPC because the α carbon and β carbon appeared to be split by the phosphorus nucleus of adenylic acid. The

Table 5. Carbon-phosphorus couplings in adenylyl-SPC (Hz).

Carbon	J_{CP}
8	3.5
9	6.1
9a	3.1
4″	8.8
5″	—[a]

[a] Poorly resolved doublet.

Fig. 2. Chemical structure of adenylyl-SPC.



chemical shifts for the carbon nuclei of the adenylyl-SPC are tabulated in Table 4. The chemical shifts for the carbon nuclei in SPC and adenylic acid were taken from the published data on the cmr spectrum of SPC[9] and adenylic acid[10]. The C–9, C–9a, C–8, and C–4″ resonances of the inactivated SPC showed doublet, and the C–5″ resonance showed poorly resolved doublet (Table 5). Therefore, the presence of these phosphorus-carbon coupling supported that the adenylyl group was attached at 9 position in the inactivated SPC molecule (Fig. 2).

Discussion

Results obtained by the incorporation of ^{14}C from [8-^{14}C]ATP into SM or SPC using four clinical isolates of *A. calcoaceticus* subsp. *anitratus* strongly suggested that these strains could produce aminoglycoside adenylyltransferase (AAD), as was previously indicated by MURRAY and MOELLERING[4].

DAVIES *et al.*[1] previously speculated that aminoglycoside 3″-adenylyltransferase, *i.e.*, AAD(3″), from *E. coli* NR73/W677 was capable of adenylylating the 9-hydroxy group of SPC. In the present studies, we show definitively that AAD(3″), isolated from a strain of *A. calcoaceticus* subsp. *anitratus* GN12313, inactivates SPC by adenylylation of the hydroxy group at the 9-C position.

To try to establish a role for plasmids in mediating the production of AAD and drug resistance in *A. calcoaceticus*, extensive efforts to transfer the drug resistance were made by filter matings, and attempts at transformation with plasmid DNAs of GN12313. No transfer of resistance was seen. Curing of plasmids with ethidium bromide was also unsuccessful. On the other hand, the transfer of SM·SPC-resistance in GN12313 to *E. coli* ML4905 was obtained in the presence of RP4. These transconjugants did not acquire the small-molecular-weight plasmids which were present in the parent strain GN12313. From cleavage analysis with restriction enzyme Sac II of the recombinant plasmid between determinant of SM·SPC-resistance and RP4, the recombinant plasmid has a segment of 3.4 Mdalton additional to RP4 DNA in the D fragment (data not shown). As BENDLER[11] and STUY[12,13] have suggested that plasmid-free antibiotic-resistant *H. influenzae* have the resistant determinants or the entire plasmid integrated into the chromosome. We questioned whether the RP4-SM·SPC recombinant plasmid formed by recombination with the determinant of SM·SPC-resistance integrated into the chromosome and RP4. On the other hand, whether the determinant of SM·SPC-resistance is transposon, or is extrachromosomal, is presently not clear. The origin of the inserted segment into RP4 is obscure at present.

References

1) BENVENISTE, R.; T. YAMADA & J. DAVIES: Enzymatic adenylylation of streptomycin and spectinomycin by R-factor-resistant *Escherichia coli*. Infect. Immunity 1: 109～119, 1970
2) SMITH, D. H.; J. A. JANJIGIAN, N. PRESCOTT & P. W. ANDERSON: Resistance factor-mediated spectinomycin resistance. Infect. Immunity 1: 120～127, 1970
3) KAWABE, H.; M. INOUE & S. MITSUHASHI: Inactivation of dihydrostreptomycin and spectinomycin by *Staphylococcus aureus*. Antimicr. Agents & Chemoth. 5: 553～557, 1974
4) MURRAY, B. E. & R. C. MOELLERING, Jr.: Evidence of plasmid-mediated production of aminoglycoside-modifying enzymes not previously described in *Acinetobacter*. Antimicr. Agents & Chemoth. 17: 30～36, 1980
5) DAVEY, R. B. & J. PITTARD: Genetic and biophysical study of R plasmids conferring sulfanilamide resistance in *Shigella* strains isolated in 1952 and 1956. J. Bacteriol. 120: 1186～1195, 1974
6) OZANNE, B.; R. BENVENISTE, D. TIPPER & J. DAVIES: Aminoglycoside antibiotics: Inactivation by phosphorylation in *Escherichia coli* carrying R factors. J. Bacteriol. 100: 1144～1146, 1969
7) TOWNER, K. J. & A. VIVIAN: RP4-mediated conjugation in *Acinetobacter calcoaceticus*. J. Gen. Microbiol. 93: 355～360, 1976
8) TOWNER, K. J. & A. VIVIAN: RP4 fertility variants in *Acinetobacter calcoaceticus*. Genet. Res., Camb. 28: 301～306, 1976
9) STROSHANE, R. M.; M. TANIGUCHI & K. L. RINEHART, Jr.: Spectinomycin biosynthesis studied by carbon

magnetic resonance spectroscopy. J. Am. Chem. Soc. 98: 3025~3027, 1976

10) DORMAN, D. E. & J. D. ROBERTS: Nuclear magnetic resonance spectroscopy: ^{13}C spectra of some common nucleotides. Proc. Nat. Acad. Sci. 65: 19~26, 1970

11) BENDLER, J. W., III.: Physical size of the donor locus and transmission of *Haemophilus influenzae* ampicillin resistance genes by deoxyribonucleic acid-mediated transformation. J. Bacteriol. 125: 197~204, 1976

12) STUY, J. H.: Plasmid transfer in *Haemophilus influenzae*. J. Bacteriol. 139: 520~529, 1979

13) STUY, J. H.: Chromosomally integrated conjugative plasmids are common in antibiotic-resistant *Haemophilus influenzae*. J. Bacteriol. 142: 925~930, 1980