A 2′-N-ACETYLATING ENZYME OF AMINOGLYCOSIDES

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We have found in our stock cultures of clinical origin *Providencia* strains that were resistant to various aminoglycoside antibiotics including lividomycin and the gentamicin C complex but sensitive to kanamycin A and a new kanamycin A derivative, BB-K8. The substrate profile of inactivation of aminoglycosides combined with structural studies of the inactivated product of 3′-deoxyparomamine, indicate that aminoglycosides were inactivated by acetylation of the amino group at the 2′-C position.

The enzymatic inactivation of aminoglycoside antibiotics is considered to be one of the mechanisms of drug resistance in bacteria. Resistance to aminoglycosides in clinical isolates of gram-positive and gram-negative bacteria is usually mediated at least by one of the enzymes capable of acetylating[1~5], phosphorylating[6,7] or adenylylating[8,9] the drugs. It is known that there are three acetyltransferases for aminoglycosides. The kanamycin-acetyltransferase reported by UMEZAWA *et al*[1]. can acetylate kanamycin-A, -B, gentamicin-C_{1a} and -C_2 but not lividomycin-A, kanamycin-C and gentamicin-C_1[2,10], gentamicin-acetyltransferase I inactivates the three components of gentamicin-C but not lividomycin-A, kanamycin-A, -B and -C[3~5].

We have found lividomycin and gentamicin-C resistant but kanamycin-A susceptible strains of *Providencia* in our stock cultures. This paper deals with the mechanism of inactivation of aminoglycoside antibiotics including lividomycin-A and gentamicin-C by these strains.

Materials and Methods

Antibiotics.

Lividomycins were supplied by Tokyo Research Laboratories, Kowa Co., Ltd. 3′-Deoxyparomamine was prepared by the method described previously[11]. Kanamycins and 3′,4′-dideoxykanamycin B[12] were kindly supplied by H. UMEZAWA, the Institute of Microbial Chemistry, Tokyo. Butirosin (A, 85% and B, 15%) and BB-K8[13] were obtained, respectively, from H. W. DION, Parke, Davis & Comp., U.S.A. and H. KAWAGUCHI, Bristol-Banyu Research Institute, Tokyo. Gentamicin C components were supplied by M. J. WEINSTEIN, The Schering Corp., U.S.A. Other antibiotics used were purchased from commercial sources.

Bacterial strains.

Providencia sp. GN1554, GN705 and GN626 were isolated from clinical specimens. *E. coli* K-12 W3630(F^-, *mal*$^-$) and ML1410(F^-, *met*$^-$, *nal*^{r}) (*nal*^{r}: resistance to nalidixic acid) were used as the recipients for the attempted transfer of drug resistance. The method of the transfer of drug resistance was described previously[14].

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Determination of drug resistance.

One loopful of 100-fold dilution (about 10^6 cells/ml) of overnight broth culture was spotted on heart infusion agar plates (Eiken, Tokyo) containing serial twofold dilutions of each antibiotic. The minimum inhibitory concentration (MIC) of each drug was scored after 18 hours of incubation at 37°C.

Preparation of the S-105 fraction.

The S-105 fraction from *Providencia* sp. was prepared by the same procedure described previously[7]. Partial purification of the enzyme from GN1554 was performed by precipitating the S-105 fraction (23 ml), which showed optical density of 90 at 280 mμ (OD unit; Hitachi 101 spectrophotometer), with ammonium sulfate at 75% saturation and the precipitate was dissolved in modified TMK solution (0.06 M KCl, 0.01 M $MgCl_2$ and 0.006 M 2-mercaptoethanol in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.8). The resulting solution (70 OD units, 10 ml) was loaded onto a Sephadex G-100 column (43×600 mm) and developed with modified TMK solution. The fractions which showed the enzyme activity were collected and used. The specific activity of the enzyme solution thus obtained was 25 fold higher than that of the original S-105 fraction.

Radioisotopic assay.

The incorporation into antibiotics of ^{14}C-acetate by the enzyme was carried out in the following reaction mixture; 20 μl of the S-105 fraction (30 OD units), 10 μl of 2 mM coenzyme A, 10 μl of 0.5 mM antibiotic, 10 μl of 40 mM adenosine triphosphate (ATP) and 10 μl of 1 μCi of α-^{14}C-sodium acetate (specific activity 48 mCi/mmole, Daiichi Pure Chemicals, Tokyo). The incorporation into antibiotics of ^{32}P from γ-^{32}P-ATP or ^{14}C from 8-^{14}C-ATP were performed in a reaction mixture containing either 0.5 μCi of γ-^{32}P-ATP (1705 mCi/mmole, Radiochemical Centre, Amersham, England) or 0.25 μCi of 8-^{14}C-ATP (50 mCi/mmole, International Chemical and Nuclear Corp. U.S.A.) instead of ^{14}C-sodium acetate. The reaction was carried out for 60 minutes at 37°C, and 10 μl of the reaction mixture was spotted onto 1 cm^2 of phosphocellulose paper (Whatman P-81), washed with distilled water and dried. Radioactivity on the paper was counted in toluene-based scintillator with a liquid scintillation counter (Packard Instrument).

Inactivation of antibiotics.

The reaction mixture consisted of 0.2 ml of the S-105 fraction (30 OD units), 0.05 ml of 1 mM antibiotic, 0.05 ml of 4 mM coenzyme A, 0.1 ml of 40 mM ATP and 0.1 ml of 40 mM sodium acetate. The reaction mixture was incubated for 60 minutes at 37°C and the reaction was stopped by heating for 5 minutes at 80°C. Residual antibiotic activity was determined by a paper disk method using *Bacillus subtilis* PCI219 as indicator. The inactivation of antibiotics by the partially purified enzyme was performed using acetyl-coenzyme A instead of coenzyme A, acetate and ATP.

Isolation of enzymatically acetylated 3′-deoxyparomamine.

The enzymatic acetylation of 3′-deoxyparomamine was carried out at 37°C for 48 hours in the following reaction mixture; 150 ml of total volume containing 307 mg of 3′-deoxyparomamine, 1.5 g of ATP, 25 mg of coenzyme A, 25 mg of creatinephosphate, 6 mg of creatinekinase, 800 mg of sodium acetate, 35 ml of the S-105 fraction (44 OD units) from *Providencia* GN1554 and modified TMK solution. The reaction was stopped by heating at 90°C for 10 minutes, the precipitate was removed by centrifugation at 4,500×g for 10 minutes, and the supernatant fluid diluted with distilled water (400 ml) and adsorbed on a CM-Sephadex (NH_4^+ form, 24×800 mm). After washing with distilled water (600 ml), the acetylated product was eluted with 0.1 N NH_4OH. The ninhydrin-positive fractions were collected and concentrated under reduced pressure. The resulting solution was then adsorbed onto an Amberlite CG-50 column (NH_4^+ form, 12×500 mm) and eluted with 0.2 N NH_4OH. The fractions which showed a ninhydrin-positive reaction were collected and concentrated. The acetylated product in the concentration was further purified by thin-layer chromatography

on Silica Gel G(Merck) with a solvent of chloroform, methanol and 2% NH_4OH(2:1:1). The product (Rf 0.63) was extracted in small volume of 0.2 N NH_4OH. The acetylated product was rechromatographed on a CM-Sephadex column (12×500 mm) and after lyophilization, a white powder (m.p. 146~152°C; 70 mg) of purified product was obtained.

Results

Detection of Acetyltransferase from *Providencia*

Three strains of *Providencia* resistant to lividomycin-A and gentamicin-C but susceptible to kanamycin-A were selected from our stock cultures. The MIC levels of various drugs toward these strains are shown in Table 1. These strains were resistant to lividomycin-A, -B, paromomycin, ribostamycin, butirosin, dideoxykanamycin-B, kanamycin-B and -C in addition to tetracycline, chloramphenicol, streptomycin and sulfonamide, but susceptible to kanamycin-A and BB-K8.

Table 1. Minimum inhibitory concentrations of various antibiotics to three strains of *Providencia*

Antibiotic	Minimum inhibitory concentration (μg/ml)		
	GN1554	GN705	GN626
Lividomycin A	>800	>800	>800
Lividomycin B	800	100	100
Paromomycin	>800	>800	>800
Ribostamycin	>800	>800	>800
Gentamicin C	25	50	100
Butirosin	>100	>100	>100
Kanamycin A	3.1	6.2	12.5
Kanamycin B	100	50	50
Kanamycin C	>200	>200	>200
Dideoxykanamycin B	50	>100	>100
BB-K8	3.1	6.2	6.2
Tetracycline	>200	>200	>200
Chloramphenicol	>200	>200	>200
Streptomycin	>200	>200	>200
Sulfonamide	>200	>200	>200

To investigate the genetic properties of the drug resistance determinant in these strains, conjugal transferability and elimination of lividomycin-resistance were examined. The lividomycin-resistance in three strains of *Providencia* could not be transferred to two *Escherichia coli* recipients; the transmission frequency being less than 10^{-8}. The lividomycin-resistance in the three strains also was not cured by treatment with acridine orange or ethidium bromide. These results suggest that the determinant governing lividomycin-resistance may not be extrachromosomal.

Next, we prepared an S-105 fraction from *Providencia* to investigate the mechanism of resistance. As shown in Table 2, the inactivations of drugs were found to parallel the incorporation of ^{14}C from labeled acetate into these drugs, and no incorporation of ^{32}P or ^{14}C from labeled ATP was seen. The MIC levels for various drugs also paralleled the inactivation

Table 2. Incroporation of isotope-labeled compounds into various aminoglycosides and their inactivation

Antibiotics	GN1554				GN705				GN626			
	^{32}P[a]	^{14}C[b]	^{14}C-Ac[c]	%[d]	^{32}P	^{14}C	^{14}C-Ac	%	^{32}P	^{14}C	^{14}C-Ac	%
Lividomycin A	83	112	1801	97	65	153	1276	91	68	161	685	54
Ribostamycin	108	208	876	68	71	140	538	55	79	146	213	31
Gentamicin C_1	77	136	992	99	86	164	1312	99	68	129	1308	99
Gentamicin C_{1a}	116	137	1257	97	160	151	1265	97	75	169	1226	96
Butirosin	75	118	1465	99	67	183	1890	100	77	188	1731	99
Kanamycin A	82	104	139	7	74	195	146	0	75	166	130	0
Kanamycin B	91	170	666	44	75	133	656	44	142	157	343	46
Kanamycin C	115	185	848	43	97	119	766	47	80	118	357	26
Dideoxykanamycin B	78	100	1117	99	77	191	1886	99	65	182	1750	98
BB-K8	89	145	79	0	72	152	90	17	79	158	67	5
Without antibiotics	126	194	84		102	143	130		104	117	86	

The S-105 fractions from GN1554, GN705 and GN626 were used. Details, see Materials and Methods.
[a] Counts per minute of ^{32}P of γ-^{32}P-ATP incorporated into antibiotics.
[b] Counts per minute of ^{14}C-labeled component of 8-^{14}C-ATP incorporated into antibiotics.
[c] Counts per minute of ^{14}C-acetate incorporated into antibiotics.
[d] Number indicates per cent of inactivation of antibiotics.

Table 3. Requirements for the inactivation of lividomycin-A by the partially purified enzyme from *Providencia* GN1554

Reaction mixture	Molar ratio of acetylcoenzyme A per lividomycin-A	Inactivation of lividomycin-A (%)
Reaction system I[a]	5	100
	2	100*
	1	100*
	0.5	52*
	0.25	30*
— acetylcoenzyme A	0	12*
— Enzyme	5	5
Reaction system II[b]		100
— Enzyme		5
— Coenzyme A		18
— S-105 fraction from ML1410		21
— ATP		21
— Acetate		25

The reaction was carried out at 37°C for 60 minutes.
* Incubation for 120 minutes at 37°C.
[a] Reaction system I consisted of 0.1 ml of partially purified enzyme (1.0 OD unit), 0.05 ml of 5 mM acetylcoenzyme A, 0.05 ml of 1 mM lividomycin-A and 0.3 ml of modified TMK solution (pH 7.8).
[b] Reaction system II consisted of 0.1 ml of partially purified enzyme (1.0 OD unit), 0.1 ml of 2 mM coenzyme A, 0.1 ml of the S-105 fraction (10 OD units) from *E. coli* ML1410, 0.05 ml of 40 mM ATP, 0.1 ml of 40 mM sodium acetate and 0.05 ml of 1 mM lividomycin-A.

of these drugs, indicating that the resistance mechanism in these strains can be accounted for by the acetylation of these drugs.

Some properties of the enzyme were investigated using a partially purified enzyme from GN1554. Table 3 shows the requirements for the inactivation of lividomycin-A. Acetyl-coenzyme A was necessary for the inactivation of lividomycin-A. One mole of lividomycin-A was completely inactivated with one mole of acetylcoenzyme A during 120 minutes of incubation at 37°C. Under the same conditions, 52% inactivation of lividomycin-A was obtained by 0.5 mole acetylcoenzyme A and 30% inactivation by 0.25 mole acetylcoenzyme A, indicating that lividomycin-A reacts with an equimolar amount of acetylcoenzyme A in the presence of the enzyme and is inactivated by the formation of acetylated product. Acetylcoenzyme A can be replaced by coenzyme A, ATP, sodium acetate and the S-105 fraction from a sensitive strain, *E. coli* ML1410. The optimal pH for lividomycin-A inactivation was near 6.5 (Fig. 1). The enzyme activity was unaffected by heating at 45°C for 5 minutes, but 62% of the activity was reduced by heating at 50°C and was completely lost on heating at 65°C for 5 minutes. The substrate specificity of the partially purified enzyme is shown in Table 4. Kanamycin-B and -C were strongly inactivated in comparison with the inactivation by the S-105 fraction, coenzyme A, acetate and ATP, and all 2′-amino antibiotics were inactivated by the partially purified enzyme in the presence of acetylcoenzyme A. But no inactivations of kanamycin-A and BB-K8 were seen.

Structure of Enzymatically Acetylated 3′-Deoxyparomamine

Enzymatically acetylated 3′-deoxyparomamine was isolated by CM-Sephadex and Amberlite CG-50 column chromatography from the reaction mixture. The product showed a single spot of Rf 0.63 on thin-layer chromatography on Silica Gel G with the solvent system of chloroform, methanol and 2% NH_4OH(2:1:1), while the Rf value of 3′-deoxyparomamine was 0.42 under the same conditions. The

Fig. 1. Optimal pH for the lividomycin-A inactivation reaction

The reaction mixture consisted of 0.1 ml of the partially purified enzyme (1.0 OD unit) from *Providencia* GN1554, 0.05 ml of 5 mM acetylcoenzyme A, 0.05 ml of 1 mM lividomycin-A and 0.3 ml of buffer solution. The reaction was carried out at 37°C for 10 minutes.

Symbols: (•), 0.067 M acetate buffer; (○), 0.1 M tris-HCl buffer

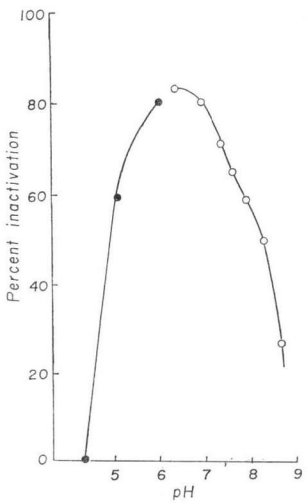


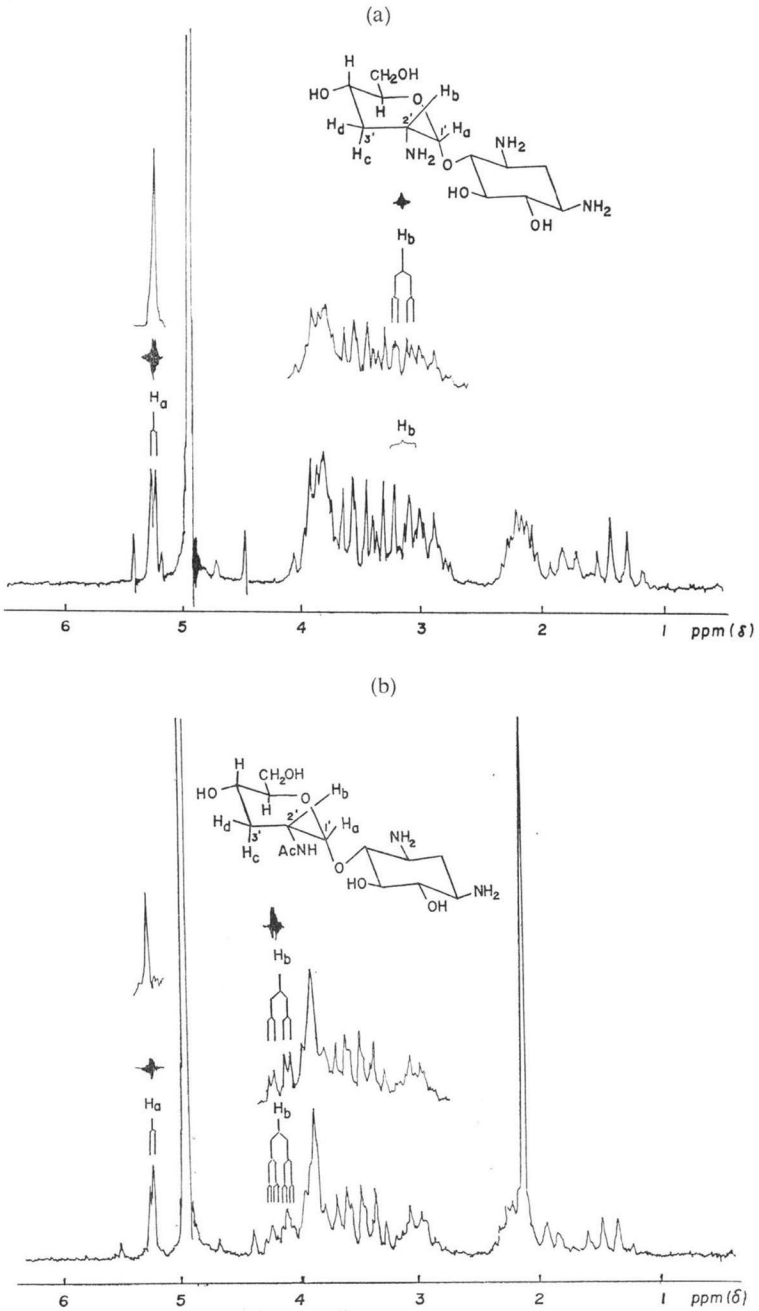
Table 4. Comparison of the inactivation of various aminoglycosides by the partially purified enzyme from *Providencia* GN1554

Antibiotic	Inactivation (%)
Lividomycin A	100
Ribostamycin	100
Gentamicin C_1	98
Gentamicin C_{1a}	95
Butirosin	99
Kanamycin A	0
Kanamycin B	94
Kanamycin C	100
Dideoxykanamycin B	98
BB-K8	0

The reaction was carried out at 37°C for 60 minutes in the reaction system I shown in Table 3.

elemental analysis of the acetylated product was as follows. Calculated for $C_{14}H_{27}N_3O_7 \cdot 5/2H_2O$: C, 44.32; H, 8.06; N, 11.08. Found: C, 44.30; H, 7.37; N, 11.06. The analysis of the primary amino groups[15] showed two in the acetylated product, while 3′-deoxyparomamine had three. An infrared spectrum of the acetylated product showed absorption bands at 1635 and 1545 cm^{-1} attributable to an amide group. In the nuclear magnetic resonance (NMR)

Fig. 2. The NMR spectra of 3′-deoxyparomamine (a) and its acetylated product (b) in D_2O at 100 MHz



spectrum of 3′-deoxyparomamine, the signals at δ 5.23 are assigned to 1′-H from published data on the NMR spectrum of various aminoglycosides[16]. Irradiation of 1′-H at δ 5.23 (J_{ab}=3.7 Hz) transformed the complicated signals between δ 3.04 and 3.25 (the center of δ 3.14) to double of doublet signals (J_{bd}=5 Hz, J_{bc}=12.5 Hz). Irradiation at δ 3.14 caused the doublet signals of 1′-H to collapse to a singlet signal (Fig. 2a). On the other hand, in that of N-acetyl-3′-deoxyparomamine the singlet signal of N-acetyl group protons was observed at δ 2.13. Irradiation at δ 5.22 (J_{ab}=3.5 Hz) attributable to 1′-H caused multiple signals (J_{ba}=3.5 Hz, J_{bd}=5.0 Hz, J_{bc}=12.5 Hz) to collapse to double-doublet type signals at δ 4.15, and irradiation at δ 4.15 resulted in a singlet signal of 1′-H (Fig. 2b). Accordingly, the signals at δ 3.14 in 3′-deoxyparomamine and δ 4.15 in the acetylated product can be assigned to the 2′-H of each compound. From the results of chemical analysis and of the low-field shift of resonance position of the 2′-H by 1.01 ppm, the structure of the enzymatically acetylated product of 3′-deoxyparomamine was concluded to be 2′-N-acetyl-3′-deoxyparomamine. This chemical structure of the acetylated product was also confirmed by the result of mass spectral analysis which showed the molecular ion peak at *m/e* 349 and a diagnostic peak at *m/e* 188 corresponding to the N-acetyl-3-deoxy-D-glucosamine fragment.

Discussion

It has been reported that lividomycin-A and kanamycin-A were inactivated by cell-free extracts from lividomycin and kanamycin-resistant strains of *E. coli*[7] and *P. aeruginosa*[17]. Recently, cross resistance between lividomycin and kanamycin has been explained by the fact that kanamycin-phosphotransferase I can phosphorylate both hydroxyl groups at the C-3 position of the D-aminoglucose moiety of kanamycin-A and at the C-5 position of the D-ribose moiety of lividomycin-A[18]. By contrast, lividomycin-A, as well as gentamicin-C was not inactivated by kanamycin-phosphotransferase II capable of phosphorylating the hydroxyl group at the C-3 position of kanamycin-A from kanamycin-resistant, lividomycin-sensitive strains, because both lividomycin-A and gentamicin-C lack the hydroxyl group at the C-3 position of the D-aminoglucose moiety[18,19]. Therefore, there are two types of resistance patterns with kanamycin and lividomycin, *i.e.*, (kanamycin, lividomycin)^r and (kanamycin)^r(lividomycin)^s. However, we have now demonstrated another type, (kanamycin-B, -C, lividomycin)^r(kanamycin-A)^s, from our stock cultures. According to the present studies, it was concluded that *Providencia* strains of this resistance type could inactivate many aminoglycosides including lividomycin-A and gentamicin-C by acetylation of the amino group at the 2′-C position. This acetyltransferase differs from the kanamycin-acetyltransferase capable of acetylating the 6′-amino group of kanamycin-A[1,2] and the gentamicin-acetyltransferase capable of acetylating the 3-amino group of gentamicin-C[3~5], with regard to the substrate specificity of these enzymes and the position of acetylation of the drugs.

BENVENISTE and DAVIES[10] previously reported that a gentamicin-acetyltransferase II from *Streptomyces spectrabilis* was capable of acetylating the 2′-amino group of gentamicin-C but not butirosin, ribostamycin, kanamycin-B and -C. After completion of this study, however, it was reported that a gentamicin-acetyltransferase II from *Providencia* could slightly inactivate butirosin, ribostamycin, kanamycin-B and -C by CHEVEREAU *et al.*[20] The characteristics of the acetyltransferase from *Providencia* GN1554 described in the present paper, therefore, is similar to those of gentamicin-acetyltransferase II in regard to the position of acetylation of antibiotics, the substrate specificity and optimal pH of the enzyme, and it may be identical with gentamicin-acetyltransferase II.

Conjugal transfer of lividomycin-A resistance and elimination of the resistance from *Providencia* strains were unsuccessful, suggesting that the determinant governing this new

type of acetyltransferase is located on the chromosome, although detailed genetic studies will be necessary to establish this.

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