PURIFICATION AND PROPERTIES OF KANAMYCIN-PHOSPHORYLATING ENZYME FROM *PSEUDOMONAS AERUGINOSA*

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Kanamycin-phosphorylating enzyme was extracted from *Pseudomonas aeruginosa* and purified by ammonium sulfate fractionation, Sephadex G-100 column and DEAE Sephadex A-50 column chromatography. This enzyme has no ATPase activity and catalyzes the reaction in which ATP reacts with kanamycin on an equimolar basis yielding kanamycin-3′-phosphate and ADP. Mg^{++} is required for the reaction and can be substituted with Mn^{++}, Zn^{++} or Co^{++}. The enzyme is protected from heat denaturation by the addition of kanamycin. Substrates having the specific structures of 6-amino-6-deoxy-α-D-glucopyranosyl, 2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl or 2-amino-2-deoxy-α-D-glucopyranosyl 2-deoxystreptamines are required for the reaction. Methyl 3-amino-3-deoxy-α-D-glucopyranoside and formycin are competitive inhibitors of kanamycin and ATP respectively. Adenosine shows stronger inhibition than ADP, AMP and adenine.

The resistance of *Escherichia coli* carrying the R factor to chloramphenicol, streptomycin and kanamycin is known to be due to the formation of enzymes inactivating these antibiotics. OKAMOTO and SUZUKI found that a cell-free system from *E. coli* carrying the R factor inactivated these antibiotics[1]. UMEZAWA *et al.* reported that *E. coli* K12 strains carrying the R factor produced two enzymes inactivating kanamycin in different ways[2]. One of the inactivated products was 6′-N-acetylkanamycin in which the amino group of the 6-amino-6-deoxy-D-glucose moiety in kanamycin was acetylated[3], and the other was kanamycin-3′-phosphate in which a hydroxy group at C-3 in the same moiety was phosphorylated[4].

In previous papers[5,6,7], we reported that most strains of *Pseudomonas aeruginosa* which were resistant to kanamycins, streptomycin and chloramphenicol produced enzymes inactivating kanamycin, kanamycin B, kanamycin C, neomycin B, paromomycin, neamine, paromamine, streptomycin and dihydrostreptomycin, and we confirmed that the inactivated kanamycin and paromamine were kanamycin-3′-phosphate and paromamine-3′-phosphate, respectively. The kanamycin-phosphorylating enzyme was separated from the streptomycin-inactivating enzyme[6].

In this papar, further purification and properties of kanamycin-phosphorylating enzyme from *P. aeruginosa*, the products and inhibitors of the enzyme reaction are reported.

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Materials and Methods

Strain of *P. aeruginosa*: *P. aeruginosa* H9 was obtained from Dr. Y. HOMMA, Institute of Medical Science, University of Tokyo. The minimum inhibitory concentrations against this strain were higher than 160 mcg/ml for chloramphenicol, 40 mcg/ml for tetracycline, higher than 160 mcg/ml for streptomycin, higher than 100 mcg/ml for neomycin high than 160 mcg/ml for kanamycin, and 6 mcg/ml for gentamicin.

Procedure for preparation of S-105 fraction: *P. aeruginosa* was grown in 0.3 % glucose broth (0.3 % glucose, 0.5 % NaCl, 1.0 % meat extract, 1.0 % peptone, at pH 7.8) for 18 hours under shaking at 37°C. The culture thus obtained was diluted about 50-fold with the same medium, cultivated under shaking to the late logarithmic phase, poured onto crushed ice, and the cells collected by centrifugation. The yield of cells was 3~4 g (wet weight) per liter. They were washed twice with TMK buffer which consisted of 60 mM KCl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 100 mM Tris hydrochloride (pH 7.8 with hydrochloric acid), homogenized in an equal volume of the same buffer and disrupted by passage through a French pressure cell (400 kg/cm^2). The extract was digested with pancreatic DNase (4 mcg/ml) and centrifuged at 10,000 × g for 30 minutes. The supernatant was again centrifuged at 105,000 × g for 2 hours and dialyzed against TMK buffer. The dialyzed solution, designated S-105 fraction, was used as starting material for the enzyme purification.

Method of assay: The reaction mixture in a final volume of 0.5 ml adjusted to pH 7.5 contained the enzyme solution, an antibiotic (substrate), ATP, 60 mM KCl, 5 mM magnesium acetate and 100 mM Tris hydrochloride. The amount of enzyme and final concentrations of antibiotic and ATP for each experiment are given in the text. The reactions were carried out at 37°C for 20 minutes in tubes placed on a reciprocating shaker unless otherwise stated. The mixture was then heated at 80°C for 5 minutes. The activity of residual antibiotic in the heated reaction mixture was determined by the disc plate method using *B. subtilis* as the test organism. The amount of enzyme is tentatively expressed as O. D. units in this paper. One O. D. unit represents the amount of protein existing in 1 ml solution whose optical density (1 cm light path) at 280 mμ is 1.0.

Purification procedures: All the procedures were conducted at below 4°C. To the S-105 fraction (4.0 O. D. units) prepared from *P. aeruginosa* H9, ammonium sulfate was added to achieve 40 % saturation. The precipitate was collected by centrifugation, dissolved in TMK buffer and dialyzed against the same buffer. The dialyzed solution was applied to a Sephadex G-100 column (20 × 550 mm), and the column developed with a buffer consisting of 100 mM KCl, 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 100 mM Tris hydrochloride (pH 7.8 with hydrochloric acid). The effluent was collected in 10 ml fractions. The fractions containing the kanamycin-phosphorylating enzyme were combined, applied to a column of DEAE Sephadex A-50 (20 × 500 mm) and fractionated by elution with a linear KCl gradient from 0.1 to 0.5 M in the buffer described above (total volume 450 ml). Active fractions were combined, and chromatography on Sephadex G-100 and DEAE Sephadex A-50 columns was repeated as described above. For the assay of enzyme activity, 0.01 or 0.1 ml of each fraction, 0.2 mM kanamycin, and 5 mM ATP were used. The specific activity of each fraction, based on enzymic activity per O. D. unit of protein, was calculated.

Paper chromatography of ATP, ADP and AMP: The reaction mixture was spotted on 40 × 40 cm paper (Toyo Roshi No. 51) and developed with isobutyric acid – 28 % ammonia – water (22 : 1 : 20) for about 16 hours[8]. As controls, authentic samples of ATP, ADP and AMP were run simultaneously. On the chromatogram, nucleotides were detected with the reagents of HANES and ISHERWOOD[9], and LEMIXEU[10] reagents. Rf values were 0.24 for ATP, 0.35 for ADP and 0.49 for AMP.

Thin-layer chromatography of substrates in the reaction mixture: A solution

containing a test material was spotted on a thin-layer of silica gel H (Merck) and developed with *n*-propanol – pyridine – acetic acid – water (51 : 20 : 6 : 24) for about 2 hours. Compounds on the chromatograms, were detected with ninhydrin, LEMIEUX[10] and RYDON-SMITH[11] reagents.

Results

The elution pattern of the kanamycin-phosphorylating enzyme from the second DEAE Sephadex A-50 column is shown in Fig. 1. The total O. D. units, specific activity, and recovery of the enzyme at each stage of purification are summarized in Table 1. A final purification of 360-fold was achieved, and the recovery of the enzymic activity was 12 %.

The amino acid composition of the purified enzyme was studied. About 1.5 mg of the purest enzyme was hydrolyzed with 6 N hydrochloric acid in a sealed glass-tube for 18 hours at 110°C. The relative molar composition was as follows: Asp 1.2, Thr 0.42, Ser 0.48, Glu 1.18, Pro 0.5, Gly 1.02, Ala 1.32, Cys positive, Val 0.74, Met 0.06, ILeu 0.44, Leu 1.28, Tyr 0.004, Phe 0.42, Lys 0.28, His 0.30 and Arg 0.72.

In a previous paper[6], we reported that ATP was necessary for the phosphorylation of kanamycin for crude preparations of the enzyme the rate of inactivation of kanamycin depended on ATP concentration up to 20 mM. At the concentrations of 30~40 mM, ATP caused a slight inhibition. However, the following experiment revealed that, using the most purified enzyme, kanamycin was completely phosphorylated by an equimolar amount of ATP. Kanamycin and ATP were mixed at various molar ratios for 90 minutes in the presence of 0.004 O. D. unit of the purified enzyme. Other conditions were as described for the assay procedure. When the kanamycin concentration was 0.2 mM, 100 % phosphorylation was achieved by 0.2 mM ATP, while 59 % phosphorylation resulted with 0.1 mM ATP. When kanamycin concentration was 0.4 mM, 100 % phosphorylation was obtained by 0.4 mM ATP, and 55 % phosphorylation and 32 % phosphorylation by 0.2 mM and 0.1 mM ATP.

As reported in a previous paper[6] using S-105 fraction as the enzyme, ADP, CTP, GTP and UTP could be substituted for ATP in the phosphorylation

Fig. 1. DEAE Sephadex A-50 column chromatography of kanamycin-phosphorylating enzyme.

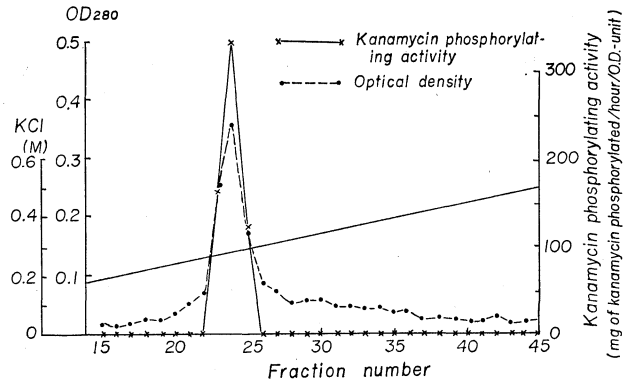


Table 1. Purification of kanamycin-phosphorylating enzyme

Fraction	Total O. D. unit	Specific activity μmoles/hr./ O. D. unit	Recovery %
1. S-105	10400	1.95	100
2. Ammonium Sulfate	1932	4.84	46
3. Sephadex G-100	174.2	44.2	38
4. DEAE Sephadex A-50	10.7	627	33
5. Sephadex G-100	6.1	672	20
6. DEAE Sephadex A-50	3.6	702	12

of kanamycin. This point was studied using the purified enzyme. The nucleotides were added at 0.2 mM and kanamycin was added at 0.2 mM to the reaction mixture. No phosphorylation of kanamycin occurred with ADP, CTP, GTP and UTP, though 100 % of kanamycin was phosphorylated with ATP.

Study of this enzyme reaction showed that ATP was converted to ADP plus kanamycin-3′-phosphate and that the enzyme had no ATPase activity without kanamycin. In the absence of enzyme, ATP and kanamycin remained unchanged in the reaction mixture. In one experiment, 0.5 ml of a reaction mixture containing 0.071 O. D. unit of pure enzyme, 10 mM ATP, 5 mM kanamycin, 60 mM KCl, 5 mM magnesium acetate, and 100 mM Tris hydrochloride (pH 7.5), after 2 hours at 37°C, 100 % inactivation of kanamycin was achieved and only ADP was detected by paper chromatography as the second reaction product. However, when kanamycin was not added to the reaction mixture, ADP was not detected and ATP concentration remained unchanged.

The effect of incubation temperature on the reaction mixture was also studied. Five mM ATP and 0.2 mM kanamycin and 0.004 O. D. unit of the enzyme in 0.5 ml of the reaction mixture were used. The reaction was conducted at 30°, 37°, 45°, 55°, 65° and 75°C. The results are shown in Fig. 2. At 65°C, the reaction proceeded only during the first 10 minutes and at 75°C no reaction occurred. The reaction proceeded most rapidly at 55°C.

The heat stability of the pure enzyme in the presence and absence of magnesium acetate, EDTA or kanamycin was studied. The mixture contained, in 0.5 ml as the final volume, EDTA at 0 or 10 mM, kanamycin at 0 or 0.4 mM, magnesium acetate at 0 or 10 mM, 0.009 O. D. unit of the enzyme and sufficient TMK buffer. It was adjusted to pH 8.0 with hydrochloric acid. The mixture was heated at various temperatures for 10 minutes, chilled, combined with an equal volume of the reaction mixture described below, and assayed for the activity in phosphorylating kanamycin. The reaction mixture (pH 7.5) consisted of 10 mM ATP, 0.4 mM kanamycin, 20 mM magnesium acetate (kanamycin and magnesium acetate were omitted when they had

Fig. 2. Effect of temperature on the phosphorylation of kanamycin. The reaction mixture was incubated at 30°, 37°, 45°, 55°, 65° and 75°C.

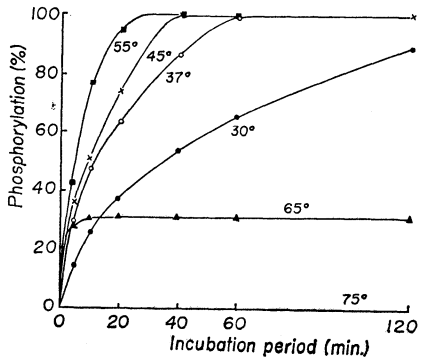
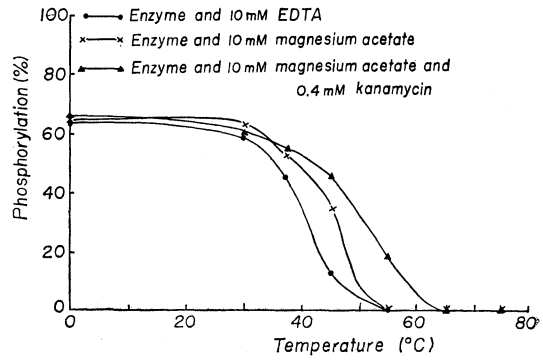


Fig. 3. The effect of kanamycin, magnesium acetate and EDTA on heat stability of kanamycin-phosporylating enzyme. Mixtures were preincubated at pH 8.0 for 10 minutes.



been added before heating) and TMK buffer. The mixtures were shaken for 20 minutes at 37°C and percentage inactivation of kanamycin determined. The results are shown in Fig. 3.

The stability of the enzyme at various pH levels was also studied. A mixture contained, in 0.5 ml as the final volume, EDTA at 0 or 10 mM, kanamycin at 0 or 0.4 mM, magnesium acetate at 0 or 10 mM, 0.010 O. D. unit of the enzyme and TMK buffer. It was adjusted with NaOH or HCl to various pHs and incubated at 37°C for 1 hour. After chilling, the pH was adjusted to 7.5. The mixture was added to an equal volume of the reaction mixture described below, and the activity of phosphorylating kanamycin determined. The reaction mixture (pH 7.5) consisted of 10 mM ATP, 0.4 mM kanamycin, 20 mM magnesium acetate (kanamycin and magnesium acetate were omitted when they had been added before preincubation) and TMK buffer. The mixture was shaken for 20 minutes at 37°C, and the results are shown in Fig. 4.

Fig. 4. pH stability of kanamycin-phosphorylating enzyme. Mixtures were preincubated at 37°C for 1 hour.

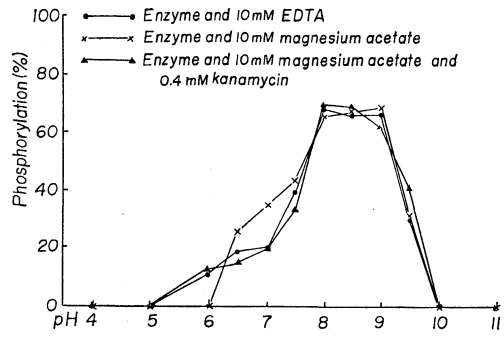


Table 2. Effect of metal compounds on the phosphorylation of kanamycin

The reaction mixture (pH 7.5) contained 0.2 mM kanamycin, 5 mM ATP, 60 mM KCl, 100 mM Tris hydrochloride, 0.002 O. D. unit of enzyme and 1 or 10 mM metal compounds. Reaction was carried at 37°C for 20 minutes.

Metal salts	Phosphorylation (%)	
	1×10^{-3} M*	1×10^{-2} M*
None	0	0
$MgCl_2$	56	69
$MnCl_2$	71	21
$CaCl_2$	6	19
$HgCl_2$	0	0
$CuSO_4$	0	0
$ZnSO_4$	81	45
$FeSO_4$	0	35
$FeCl_3$	0	0
$CoCl_2$	90	29
$NiCl_2$	0	0
$LiSO_4$	0	0
K_2CrO_4	0	0
$Al_2(SO_4)_3$	13	0

* Concentrations of metal salts

As described in Materials and Methods, the complete reaction mixture contained magnesium acetate. Therefore, effects of other metal compounds on the reaction were studied. A candidate compound substituting for magnesium acetate was examined at 1 or 10 mM for its ability to support the reaction. The reaction mixture (pH 7.5) contained 0.2 mM kanamycin, 5 mM ATP and 0.002 O. D. unit of the enzyme. Other conditions were the same as described for the assay. It was found that Mg^{++}, Mn^{++}, Zn^{++} and Co^{++} supported the reaction but Ca^{++}, Fe^{++} and Al^{+++} did so only slightly. The results are shown in Table 2.

Inhibition of the enzyme by various compounds was studied. The reaction mixture contained 0.2 mM kanamycin, 5 mM ATP, 0.001 O. D. unit of enzyme and an inhibitor at various concentrations. Other conditions were the same as described for the assay method. The following percentage inhibition was observed with compounds known to inhibit metal enzymes: EDTA, 96% at 5×10^{-3} M, 0% at 5×10^{-4} M; KCN,

27 % at 10^{-2} M, 10 % at 10^{-3} M; NaN_3, 54 % at 10^{-2} M, 0 % at 10^{-3} M. The compounds which were known to inhibit enzymes containing sulfhydryl group showed the following percentage inhibition: monoiodoacetic acid, 37 % at 10^{-2} M, 15 % at 10^{-3} M; ω-chloroacetophenone, 34 % at 5×10^{-3} M, 28 % at 5×10^{-4} M; *p*-chloromercuribenzoate, 100 % at 5×10^{-3} M, 100 % at 5×10^{-4} M; oxidized glutathione, 60 % at 4×10^{-3} M, 28 % at 4×10^{-4} M. Reduced glutathione showed no inhibition at 5×10^{-4} M. Strong inhibition by $CdCl_2$ (79 %), $SnCl_2$ (77 %), $BaCl_2$ (69 %) and HgCl (71 %) and $CuSO_4$ (100 %) was shown at 3×10^{-3} M. CsCl showed no inhibition and $SbCl_3$ showed 26 % inhibition at 3×10^{-3} M. N-Bromosuccinimide (100 % inhibition at 10^{-4} M), BrCN (66 % at 5×10^{-3} M, 0 % at 5×10^{-4} M), and iodine (100 % at 10^{-3} M, 42 % at 10^{-4} M) which were known to react with peptides showed inhibition. $NaNO_2$ showed slight inhibition (18 % at 10^{-2} M) and diisopropyl fluorophosphate showed no inhibition at 5×10^{-3} M.

Inhibitory effects of sugars, derivatives of sugars and compounds related to ATP were studied. The reaction mixture contained 0.2 mM of kanamycin, 5 mM of ATP, 0.001 O. D. unit of the enzyme and 0.2, 0.8 or 3.2 mM of inhibitor. Other conditions were the same as described for the assay. The following compounds showed no inhibition: D-glucose, D-galactose, D-mannose, D-talose, mannitol, sorbitol, inositol, dulcitol, D-rhamnose, D-arabinose, D-xylose, sucrose, maltose, lactose, D-raffinose, salicin, mycaminose, methyl α-D-glucopyranoside, methyl α-D-mannopyranoside, 2-amino-2-deoxy-D-glucose, D-glucosamic acid, D-glucosamine oxime, N-acetyl-D-glucosamine, 2-deoxystreptamine, 2-amino-2-deoxy-D-galactose, mannosyl glucosaminide[12], trehalose, trehalosamine[13], kasugamycin, kasuganobiosamine[14], methyl α-DL-kasugaminide[15], tetra-N-acetylkanamycin, 5-O-(β-D-talopyranosyl)-3-N-methyl-2-deoxystreptamine[16]. Slight inhibition was observed with the following compounds (the values in parentheses indicate the inhibition percentages and the concentrations of the inhibitors): 3-amino-3-deoxy-D-glucose (30 % at 3.2 mM), 6-amino-6-deoxy-D-glucose (18 % at 3.2 mM), methyl 2-amino-2-deoxy-β-D-glucopyraside (20 % at 0.8 mM, 24 % at 3.2 mM), methyl 6-amino-6-deoxy-α-D-glucopyranoside (18 % at 0.8 mM, 22 % at 3.2 mM), 6′-N-acetylkanamycin[3] (24 % at 0.8 mM, 35 % at 3.2 mM), 6-O-(3-amino-3-deoxy-α-D-glucopyranosyl)-2-deoxystreptamine[17] (18 % at 0.2 mM, 25 % at 0.8 mM, 36 % at 3.2 mM). The following compounds showed relatively strong inhibition: methyl 3-amino-3-deoxy-α-D-glucopyranoside (13 % at 0.2 mM, 45 % at 0.8 mM, 67 % at 3.2 mM), kanamycin-3′-phosphate[2] (13 % at 0.2 mM, 41 % at 0.8 mM, 70 % at 3.2 mM), 4-O-(6-amino-6-deoxy-α-D-glucopyranosyl)-2-deoxystreptamine[18] (30 % at 0.2 mM, 66 % at 0.8 mM and 100 % at 3.2 mM). Inhibition was observed with the following compounds related to ATP: ADP (25 % at 0.2 mM, 37 % at 0.8 mM, 69 % at 3.2 mM), AMP (19 % at 0.2 mM, 30 % at 0.8 mM, 58 % at 3.2 mM), adenosine (7 % at 0.2 mM, 48 % at 0.8 mM, 100 % at 3.2 mM), formycin[19] (23 % at 0.2 mM, 46 % at 0.8 mM, 61 % at 3.2 mM) and formycin B[20] (13 % at 0.2 mM, 27 % at 0.8 mM, 39 % at 3.2 mM).

With methyl 3-amino-3-deoxy-α-D-glucopyranoside and formycin, the effects of concentration of substrate and inhibitors on the reaction velocity were analyzed by the LINEWEAVER-BURK plot[21]. Reaction velocities were determined in the presence and absence of an inhibitor as the concentration of kanamycin was varied. The

Fig. 5. LINEWEAVER-BURK plot of kanamycin concentration against rate of kanamycin phosphorylation with or without 2 mM of methyl-3-amino-3-deoxy-α-D-glucopyranoside. 1/V, (μmoles of phosphorylated kanamycin/hour/O. D. unit)$^{-1}$; 1/S, (mM of kanamycin)$^{-1}$.

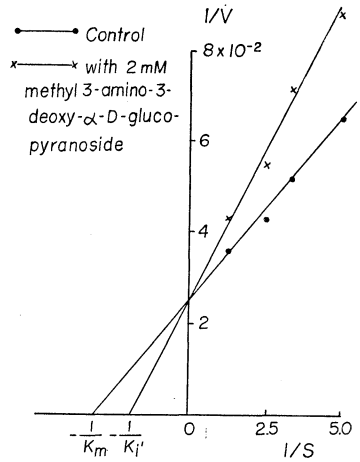
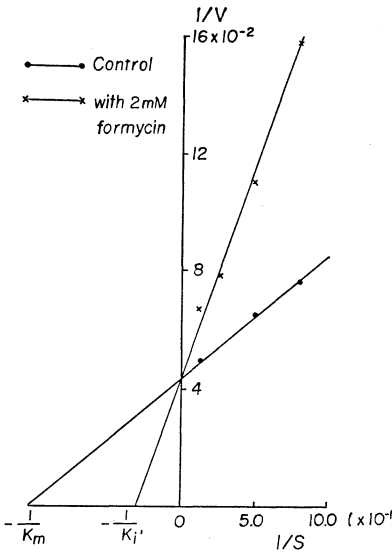


Fig. 6. LINEWEAVER-BURK plot of ATP concentration againt rate of kanamycin phosphorylation with or without 2 mM of formycin. 1/V, (μ moles of phosphorylated kanamycin/hour/O. D. unit)$^{-1}$; 1/S, (mM of ATP)$^{-1}$.



reaction system contained 5 mM ATP, 0.004 O. D. unit of enzyme, 0 or 2 mM methyl 3-amino-3-deoxy-α-D-glucopyranoside and various concentrations of kanamycin. Other conditions were as described for the method of assay. The results are shown in Fig. 5. In the absence of inhibitor, the *Km* value calculated for kanamycin was 3.24×10^{-4} M and, in the presence of inhibitor, the *Ki'* value calculated was 5.26×10^{-4} M. In the presence or absence of inhibitor, V_{max} was calculated to be 42 μmoles/hour/O. D. unit. Reaction velocities were determined also when the concentration of ATP was varied and that of kanamycin kept constant in the presence and absence of formycin[19], an adenosine analogue. The reaction system contained 0.2 mM kanamycin, 0.004 O. D. unit of enzyme, 0 or 2 mM formycin and various concentrations of ATP. It was incubated for 10 minutes. Other conditions were the same as described for the method of assay. The results are shown in Fig. 6. In the abesence of the inhibitor, the *Km* value calculated for ATP was 9.70×10^{-4} M and the *Ki'* value calculated for formycin was 3.34×10^{-3} M. In the presence or absence of the inhibitor, V_{max} was calculated to be 24 μmoles/hour/O. D. unit.

Substrate specificity of the kanamycin-phosphorylating enzyme was studied. The reaction mixture (0.5 ml, pH 7.5) containing 10 mM ATP, 20 mM KCl, 5 mM magnesium acetate, 0.013 O. D. unit of the enzyme, 4 mM substrate and 10 mM Tris hydrochloride was incubated at 37°C for 1 hour. The rate of phosphorylation of each substrate was determined by thin-layer chromatography or by the disc-plate method. Kanamycin, kanamycin B, kanamycin C, kanamycin-6''-phosphate[22], 3''-deamino-3''-hydroxykanamycin (NK 1001 substance, a kanamycin analogue which contained D-glucose instead of 3-amino-3-deoxy-D-glucose in kanamycin)[23,24], neamine, paromamine, 4-O-(6-amino-6-deoxy-α-D-glucopyranosyl)-2-deoxystreptamine[18], neomycin B, paro-

momycin and zygomycin A_2 (identical with paromomycin II) were completely phosphorylated, while 6′-N-acetylkanamycin[3] was phosphorylated only slightly. In contrast, streptomycin, dihydrostreptomycin, hydroxystreptomycin, gentamicin complex[25], kasugamycin, streptothricin (A-249 substance)[26], leucomycin, spiramycin, 5-O-(β-D-talopyranosyl)-3-N-methyl-2-deoxystreptamine[27], tetra-N-acetylkanamycin, 2-amino-2-deoxy-D-glucose, 3-amino-3-deoxy-D-glucose, 6-amino-6-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, N-acetyl-D-glucosamine, methyl 2-amino-2-deoxy-β-D-glucopyranoside, methyl 3-amino-3-deoxy-α-D-glucopyranoside, methyl 6-amino-6-deoxy-α-D-glucopyranoside, mannosyl glucosaminide[12], trehalosamine[13], trehalose, sucrose, maltose, lactose, D-glucose, D-galactose, D-mannose, D-talose, D-arabinose, D-xylose, D-rhamnose, raffinose, salicin, mannitol, inositol were not phosphorylated.

Discussion

The experiments show that there is an absolute requirement of ATP for the enzyme reaction, and it reacts with kanamycin on mole-mole basis yielding kanamycin-3′-phosphate and ADP, and that this enzyme has no ATPase activity. Therefore, the reaction can be represented as follows:

$$\text{Kanamycin} + \text{ATP} \xrightarrow{\text{enzyme}} \text{Kanamycin-3′-phosphate} + \text{ADP}$$

The enzyme was least stable to heating in the presence of EDTA and more stable in the presence of magnesium acetate and kanamycin than in the presence of magnesium acetate. Thus, kanamycin has a protective effect on the enzyme, suggesting that kanamycin binds with the enzyme in the absence of the second substrate (ATP).

It was reported in a previous paper[6], using the S-105 fraction as the enzyme, that the optimum pH for the reaction was 7.5. This was found also using the purest sample of enzyme. However, it was found that at 37°C the enzyme was more stable at pH 7.8～9.1 than at pH 7.5. The stability at various pHs was not influenced by kanamycin and magnesium acetate.

The whole kanamycin structure is not required for the enzyme reaction, because 6-amino-6-deoxy-α-D-glucopyranosyl, 2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl and 2-amino-2-deoxy-α-D-glucopyranosyl 2-deoxystreptamines were phosphorylated as well as the kanamycins. 2-Deoxystreptamine is the moiety required for the enzyme reaction, because methyl 6-amino-6-deoxy-α-D-glucopyranoside was not phosphorylated. These results suggest that the 3-amino-3-deoxy-D-glucose moiety of the kanamycins is not involved in binding of the antibiotics with the enzyme. This is supported by another observation that a kanamycin analogue in which the 3-amino-3-deoxy-D-glucose moiety is replaced by D-glucose was phosphorylated as well as kanamycins.

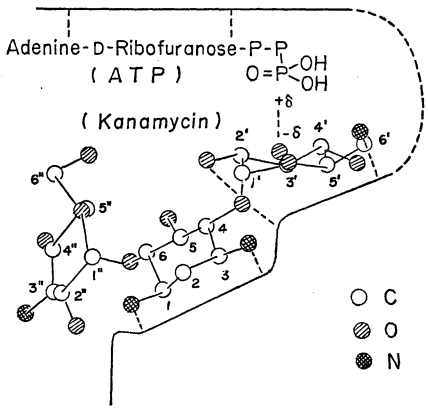
For phosphorylation of the hydroxyl group, it is considered that the hydroxyl group must be brought close to the phosphorus atom which has gained a more positive charge. Then the 6-amino-6-deoxy-D-glucose, 2-amino-2-deoxy-D-glucose or 2,6-diamino-2,6-dideoxy-D-glucose moiety linked to 2-deoxystreptamine is considered to be involved in binding with the enzyme which brings the hydroxyl group on C-3 close the terminal phosphorus atom of ATP. In support of this, methyl 6-amino-6-deoxy-α-D-glucopyranoside was not a substrate but an inhibitor. However, inhibition by 2-deoxystreptamine was not experimentally proven. In view of the substrate specificity of the enzyme, binding of 2-deoxystreptamine moiety with the enzyme is considered to be weaker than that of the aminohexose moiety. In the binding of aminohexoses with the enzyme, the amino or hydroxyl group on C-6 must be involved, because 6′-N-acetylkanamycin was only slightly phosphorylated.

The competitive relation of methyl 3-amino-3-deoxy-α-D-glucopyranoside with kana-

mycin indicates that this glucoside competes with the 6-amino-6-deoxy-D-glucose moiety of kanamycin. Methyl 3-amino-3-deoxy-α-D-glucopyranoside showed stronger inhibition than methyl 6-amino-6-deoxy-α-D-glucopyranoside. The amino group on C-3 might enhance the strength of the binding of the glucoside with the enzyme.

Among the relatively strong ATP analogues which inhibited the reaction, adenosine showed stronger inhibition than ADP, which was a reaction product. The phosphate groups of ADP may not have a strong role in binding with the enzyme. Inhibition by adenine and the stronger inhibition by adenosine suggest that both the base moiety and the ribose moiety are concerned in binding with the enzyme. Angustmycin A, which has a methylene group instead of a carbinol and hydrogen on C-4 of the ribose of adenosine, showed no inhibition. This suggests that the 5'-hydroxyl group of adenosine is strongly concerned in binding with the enzyme. Formycin which is an analogue of adenosine in which the 8-C and 9-N atoms are replaced by N and C respectively, showed weaker inhibition than adenosine. This indicates binding of the adenine moiety of ATP with the enzyme. Formycin B which has a hydroxyl group instead of the amino group of formycin showed much weaker inhibition than formycin, suggesting that the amino group of adenosine is concerned with binding to the enzyme.

Fig. 7. Scheme of the enzyme reaction.



The experimental results discussed above can be explained by the enzyme reaction scheme shown in Fig. 7. In this scheme, the stereostructure shown by X-ray crystallography of kanamycin monosulfate[26] has been utilized. The positive charge of the terminal phosphorus atom of ATP may be increased by the effect of metal ions which were required for the enzyme reaction.

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