AMINOGLYCOSIDE 3′-PHOSPHOTRANSFERASES I AND II IN *PSEUDOMONAS AERUGINOSA*

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Aminoglycoside 3′-phosphotransferases I and II in three strains of *Pseudomonas aeruginosa* were studied in comparison with those in two strains of R factor-carrying *Escherichia coli*. The strain TI-13 of *P. aeruginosa* produced the former and strain H-9 the latter. Strain B-13 produced the both enzymes. The 3′-phosphotransferases of type I in *P. aeruginosa* TI-13, B-13 and *E. coli* K12 J5 R11-2 were different from each other in chromatographic behavior, molecular weight, pH optimum, and Ki. The 3′-phosphotransferase of type II in *P. aeruginosa* H-9 and *E. coli* JR66/W677 showed the same behavior.

In a previous paper[1], we reported the presence of two kanamycin-neomycin phosphotransferases in R factor-carrying *Escherichia coli* strains. These enzymes can be called aminoglycoside 3′-phosphotransferases I [APH(3′)-I] and II [APH(3′)-II]. APH(3′)-I transfers the terminal phosphate of adenosine triphosphate to the 3′-hydroxyl group of kanamycins, neomycins, paromomycins and ribostamycin, and to the 5″-hydroxyl group (the 5-hydroxyl group of ribose moiety) of lividomycins and 3′, 4′-dideoxyribostamycin, but not to the 3′-hydroxyl group of butirosins. APH(3′)-II transfers phosphate to the 3′-hydroxyl group of kanamycins, neomycins, paromomycins, ribostamycin and also butirosins, but not to the 5″-hydroxyl group of lividomycins and 3′, 4′-dideoxyribostamycin.

As first reported by us[2], pseudomonas strains contain aminoglycoside phosphotransferases and we reported on the purification of an enzyme in *P. aeruginosa* H-9[3]. After finding the presence of APH(3′)-I and APH(3′)-II in R factor-carrying *E. coli*, we found both of them also in pseudomonas and, moreover, APH(3′)-I in pseudomonas was divided into two types by affinity chromatography. In this paper, we report substrate requirements, affinity-chromatographic behavior, molecular weights, optimal pH and inhibition constants of APH(3′)-I and APH(3′)-II in pseudomonas.

Materials and Methods

Antibiotics: Kanamycin, 3′, 4′-dideoxykanamycin B (DKB), ribostamycin and formycin were supplied by Meiji Seika Kaisha, Ltd., butirosin A by Bristol-Banyu Research Institute, and lividomycin A by Kowa Co.

Bacterial strains: *P. aeruginosa* B-13 was supplied by Dr. J. CARVAIS, Lab. Roger Bellon, Paris. *P. aeruginosa* TI-13[4], H-9, *E. coli* K12 J5 R11-2[5] and JR66/W677[6] were described in previous papers.

Preparation of crude enzymes: Organisms were grown at 37°C for 16 hours in a medium (pH 7.4) containing 1 % peptone, 0.5 % meat extract and 0.3 % sodium chloride. A 2 % inoculum was added to the same medium cantaining 10 μg/ml of kanamycin. After 6 hours of culture at 37°C, the late-logarithmic phase cells were harvested by centrifugation. The cells

were washed twice with buffer A which consisted of 20 mM tris-hydrochloric acid buffer (pH 7.2), 10 mM magnesium acetate, 60 mM potassium chloride and 10 mM 1, 4-dithiothreitol, and suspended in an equal weight of buffer A. The cell suspension was passed through a French pressure cell (American Instrument Co., Silver Spring, Md.) under a pressure of 1,200 kg/cm^2, and the disrupted-cell suspension was ultracentrifuged at 100,000g for 90 minutes. The supernatant thus obtained was designated S-100 solution.

Assay of phosphotransferase activity by determining the residual antibiotic: The reaction mixture (1.0 ml) containing 0.05 mM antibiotic, 4.0 mM adenosine triphosphate, 10 mM magnesium acetate, 60 mM potassium chloride, 10 mM 1, 4-dithiothreitol, 100 mM tris-hydrochloric acid buffer (pH 7.2), and 0.1 ml of the enzyme solution (in exceptional case testing a weak enzyme solution, 0.4 ml was added) was incubated for 15, 30, 60 or 120 minutes at 37°C. The reaction was terminated by placing the tube in a boiling water bath for 2 minutes. The enzymatic activity was estimated by the assay of the residual antibiotic in the reaction mixture. The quantity of the antibiotic was determined by a disc plate method using *Bacillus subtilis* PCI 219 as the test organism.

Affinity chromatography with kanamycin-, lividomycin A- and DKB-Sepharose 4B: Sepharose 4B which contained covalently-bound kanamycin, lividomycin A or DKB was prepared for affinity chromatography as described in a previous paper[5]. The column (0.75×6.6 cm) of kanamycin-, lividomycin A- or DKB-Sepharose 4B was equilibrated with buffer A. A S-100 solution (1~5 ml) was applied to the column and the column was washed with 25 ml of buffer A. The enzyme adsorbed on the column was eluted with a linear gradient between 50 ml (or 60 ml) of buffer A and buffer A containing 1 M (or 1.2 M) sodium chloride at a flow rate of 25 ml per hour at 4°C. The eluate was cut into each approximately 1.3-ml fraction. Enzymatic activity in each fraction was determined by the method described above.

Estimation of molecular weight by gel-filtration: The enzymatically active fractions eluted from the column of kanamycin-Sepharose 4B were combined and concentrated to less than 2 ml with the aid of a collodion bag. The concentrate (1 ml) was applied to a column (1.76×41 cm) of Sephadex G-100 equilibrated with buffer A. The enzyme was eluted with the buffer at a flow rate of about 15 ml per hour at 4°C. Column effluents were collected in 1.1-ml fractions. The molecular weight of the enzyme was estimated according to the method of ANDREWS[7]. The standard proteins used were cytochrome C (mol. wt. 12,500), chymotrypsinogen A (25,000), ovalbumin (45,000) and bovine serum albumin (67,000) (purchased from Boehringer Mannheim). Blue dextran was eluted with 27.5 ml. These standard proteins were determined by measurement of ultraviolet absorption at 280 nm. Enzymatic activity was determined by the method described above.

Method of testing enzymatic activity using γ-^{32}P-adenosine triphosphate: The reaction mixture (250 μl) consisted of 1~400 μM of an antibiotic, 10.0~1,600 μM adenosine triphosphate, 0.005~0.5 μCi of γ-^{32}P-adenosine triphosphate (5.78 Ci/mmole, purchased from New England Nuclear, Boston, Mass.), 25 μl of the proper buffer solution (250 mM, described below), 10 mM magnesium acetate, 60 mM potassium chloride, 10 mM 1, 4-dithiothreitol and 25~100 μl of the enzyme solution. The reaction mixture was incubated at 37°C for 15~60 minutes. Thereafter it was diluted with 14 ml of water and passed through a column of Amberlite CG-50 resin (NH_4^+ form, 1 ml), and the column was washed with 14 ml of water. The phosphorylated antibiotic on the column was eluted with 3 ml of 4 N aqueous ammonia into a scintillation vial, and 8 ml of BRAY's scintillator was added. The radioactivity (dpm) corresponding to the amount of the phosphorylated antibiotic was counted by a liquid scintillation system (Aloka LSC-653).

Optimal pH: The reaction mixture described in the previous section was adjusted to a specific pH by the following buffers: acetate buffer (pH 4.2~6.3), phosphate buffer (pH 5.6~8.2) and tris-hydrochloric acid buffer (pH 7.2~9.0). The effect of pH on the enzyme reaction was determined by using γ-^{32}P-adenosine triphosphate. Enzyme solutions purified by affinity chromatography on DKB-Sepharose 4B were employed.

Michaelis constant (Km): Km for an antibiotic or adenosine triphosphate was determined from LINEWEAVER-BURK plots. In the case of determination of Km for an antibiotic, 1,600 μM of adenosine triphosphate was used. Km for adenosine triphosphate was determined as follows: 40 μM of lividomycin A was used as the substrate for APH(3′)-I, and 100 μM of butirosin A for APH(3′)-II. Enzyme solutions purified by affinity chromatography on DKB-Sepharose 4B were used and the enzymatic activity was determined by the radioisotope method.

Inhibition constant (Ki): Ki of DKB against the substrate antibiotic and Ki of formycin against adenosine triphosphate were obtained from LINEWEAVER-BURK plots of the data from a reaction mixture containing 0.05~100 μM of DKB or 100~2,000 μM of formycin.

Results and Discussion

The phosphorylated products of kanamycin, lividomycin A and butirosin A were isolated by means of the immobilized enzymes prepared from the S-100 solutions of *P. aeruginosa* TI-13, H-9 and B-13 as described previously[8]. The products of kanamycin and lividomycin A phosphorylated by the immobilized enzyme of *P. aeruginosa* TI-13 were identified as kanamycin 3′-phosphate and lividomycin A 5″-phosphate, respectively, but the enzyme from this strain did not phosphorylate butirosin A. Thus, it was shown that the enzyme from strain TI-13 is a APH(3′)-I type. The immobilized enzyme of *P. aeruginosa* H-9 phosphorylated the 3′-hydroxyl group of kanamycin and also the 3′-hydroxyl group of butirosin A, but did not phosphorylate the 5″-hydroxyl group of lividomycin A. Thus, the enzyme of this strain is classed as APH(3′)-II.

Table 1. Affinity chromatography of APH(3′)-I and APH(3′)-II

Source	APH(3′)	Eluted with NaCl (M)		
		Kanamycin-Sepharose 4B	Lividomycin A-Sepharose 4B	DKB-Sepharose 4B
P. aeruginosa TI-13	I	0.28	0.68	0.41
P. aeruginosa B-13	I	0.57	1.01	0.76
E. coli K12 J5 R11-2	I	0.67	0.86	0.91
P. aeruginosa H-9	II	0.66	0.52	0.40
P. aeruginosa B-13	II	0.45	0.50	0.25
E. coli JR66/W677	II	0.55	0.52	0.36

Table 2. Purification of APH(3′)-I and APH(3′)-II by affinity chromatography using a column of DKB-Sepharose 4B

Source	APH(3′)	Specific activity, U/mg protein		Recovery %	Fold
		S-100	Purified enzyme		
P. aeruginosa TI-13	I	0.03	1.39	75.0	50.0
P. aeruginosa B-13	I	0.03	1.75	56.7	63.6
E. coli K12 J5 R11-2	I	0.71	20.0	55.0	28.3
P. aeruginosa H-9	II	0.06	2.78	73.0	50.5
P. aeruginosa B-13	II	0.01	0.28	64.4	39.6
E. coli JR66/W677	II	1.03	41.67	72.2	40.7

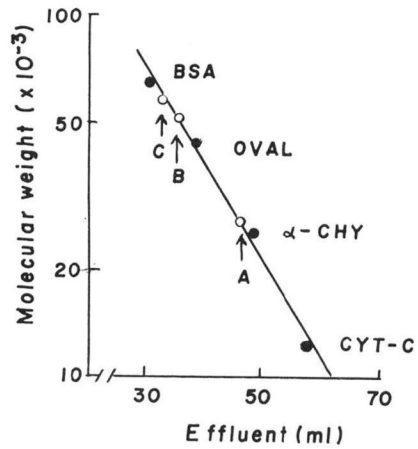
U for APH(3′)-I or APH(3′)-II: One unit is defined as the amount of enzyme which phosphorylates 1 μmole of lividomycin A or butirosin A per 1 hour.

Table 3. Molecular weights of APH(3′)-I and APH(3′)-II.

Source	APH(3′)	Molecular weight ($\times 10^3$)
P. aeruginosa TI-13	I	27
P. aeruginosa B-13	I	62
E. coli K12 J5 R11-2	I	54
P. aeruginosa TI-13	II	27
P. aeruginosa B-13	II	27
E. coli JR66/W677	II	27

Fig. 1. Estimation of molecular weights by gel-filtration on a Sephadex G-100 column

Standard proteins: Bovine serum albumin (BSA), ovalbumin (OVAL), α-chymotrypsinogen (α-CHY) and cytochrome C (CYT-C). Arrows indicate the elution volume of each enzymatic activity. A: APH(3′)-I of *P. aeruginosa* TI-13, and APH(3′)-II of *P. aeruginosa* H-9, B-13 and *E. coli* JR66/W677, B: APH(3′)-I of *E. coli* K12 J5 R11-2, C: APH(3′)-I of *P. aeruginosa* B-13.



The immobilized enzyme prepared from the S-100 solution of *P. aeruginosa* B-13 phosphorylated kanamycin, lividomycin A and also butirosin A. Two enzymes were separated by affinity chromatography using kanamycin-, lividomycin A- or DKB-Sepharose 4B and it was confirmed that strain B-13 forms both APH(3′)-I and APH(3′)-II.

The concentrations of sodium chloride for elution of APH(3′)-I and APH(3′)-II from pseudomonas strains TI-13, B-13, H-9 and R factor-carrying *E. coli* strains K12 J5 R11-2 and JR66/W677 are shown in Table 1. The chromatographic behavior indicates the presence of three types of APH(3′)-I. However, there was not a significant difference in the sodium chloride concentration for elution of APH(3′)-II in all strains. As shown in Table 2, one-step affinity chromatography gave 28.3~63.6 fold purification in good yield.

Molecular weights of these enzymes estimated by the gel-filtration method are shown in Fig. 1 and Table 3. APH(3′)-I enzymes from two strains of pseudomonas (TI-13, B-13), and R factor-carrying *E. coli* (K12 J5 R11-2) were found to have different molecular weights. However, APH(3′)-II from two pseudomonas strains (H-9, B-13), and R factor-carrying *E. coli* (JR66/W677) had the same molecular weight.

The optimal pH for APH(3′)-I from the above three strains and for APH(3′)-II from two strains is shown in Table 4. The data indicate the presence of three types of APH(3′)-I. The

Table 4. Optimal pH for APH(3′)-I and APH(3′)-II

Source	APH(3′)	Optimal pH			
		Substrate antibiotic			
		Kanamycin	Ribostamycin	Lividomycin A	Butirosin A
P. aeruginosa TI-13	I	8.1	8.1	8.1	—
P. aeruginosa B-13	I	7.5	7.5	7.8	—
E. coli K12 J5 R11-2	I	5.1	4.8	5.1	—
P. aeruginosa H-9	II	8.4	8.7	—	8.4
E. coli JR66/W677	II	8.4	8.4	—	8.1

values of optimal pH for APH(3′)-I of *P. aeruginosa* TI-13, B-13 and *E. coli* K12 J5 R11-2 were 8.1, 7.5~7.8, and 4.8~5.1, respectively. On the other hand, the optimal pH values of APH(3′)-II from *P. aeruginosa* H-9 and *E. coli* JR66/W677 were not significantly different. Reactions of APH(3′)-I and APH(3′)-II proceeded more rapidly in phosphate buffer than in the other buffer solutions, and we could not find an optimal pH of these enzymes in phosphate buffer: the reactions in phosphate buffer proceeded at the same rate within the range of 6.2 and 7.4.

The Km values of APH(3′)-I for kanamycin, ribostamycin and lividomycin A and those of APH(3′)-II for kanamycin, ribostamycin and butirosin A, and the Km values of both enzymes for adenosine triphosphate are shown in Table 5. Of the three antibiotics, lividomycin A was the best substrate for APH(3′)-I. Phosphorylation of the 5″-hydroxyl group of lividomycin A is more rapid than that of the 3′-hydroxyl group of kanamycin or ribostamycin. It was also observed[1] that phosphorylation of the 5″-hydroxyl group of 3′, 4′-dideoxyribostamycin and lividomycin B proceeded more rapidly than that of the 3′-hydroxyl group of ribostamycin and paromomycin. There was no significant difference in Km values of APH(3′)-I obtained from different strains. On the action of APH(3′)-I, substrate inhibition by kanamycin, ribostamycin and lividomycin A was observed at a concentration of 100 μM. The Km values of APH(3′)-II from two strains were also the same. There was no substrate inhibition (400 μM) of the action of APH(3′)-II. It is notable that, in all cases, Km for adenosine triphosphate was significantly higher than Km for the antibiotics.

DKB which lacks the 3′-hydroxyl group is not a substrate for these enzymes and exhibited strong inhibition. The Ki values of DKB against the phosphorylation of lividomycin A by APH(3′)-I and butirosin A by APH(3′)-II are shown in Table 6. DKB showed competitive-

Table 5. Km of APH(3′)-I and APH(3′)-II

Source	APH(3′)	Km×10^{-6} M				
		Substrate antibiotic				ATP
		Kanamycin	Ribostamycin	Lividomycin A	Butirosin A	
P. aeruginosa TI-13	I	50.0	67.0	12.6	—	606
P. aeruginosa B-13	I	83.3	56.0	17.2	—	541
E. coli K12 J5 R11-2	I	47.8	52.4	9.8	—	889
P. aeruginosa H-9	II	71.4	28.6	—	71.3	187
E. coli JR66/W677	II	81.5	28.6	—	77.2	213

Table 6. Ki Values of 3′,4′-dideoxykanamycin B against a substrate antibiotic and of formycin against ATP.

Source	APH(3′)	Substrate antibiotic	Ki×10^{-6}M	
			3′, 4′-Dideoxykanamycin B	Formycin
P. aeruginosa TI-13	I	Lividomycin A	1.31	1,650
P. aeruginosa B-13	I	Lividomycin A	2.68	426
E. coli K12 J5 R11-2	I	Lividomycin A	0.06	997
P. aeruginosa H-9	II	Butirosin A	5.23	861
E. coli JR66/W677	II	Butirosin A	4.95	475

inhibition of phosphorylation of the antibiotics by APH(3′)-I or APH(3′)-II. The Ki values of DKB against the phosphorylation of lividomycin A by APH(3′)-I of *P. aeruginosa* TI-13, B-13 and *E. coli* K12 J5 R11-2 were 1.31, 2.68 and 0.06×10^{-6} M, respectively. However, Ki values of DKB against the phosphorylation of butirosin A by APH(3′)-II of *P. aeruginosa* H-9 and *E. coli* JR66/W677 were almost the same. Formycin, a nucleoside antibiotic with a C-glycoside linkage, showed competitive-inhibition against adenosine triphosphate in the action of APH(3′)-I and APH(3′)-II, though the inhibition was very weak.

From the above data, it can be concluded that *P. aeruginosa* strains produce APH(3′)-I and APH(3′)-II which appear singly or together in a strain, and that APH(3′)-I enzyme from of two strains of *P. aeruginosa* and from an R factor-carrying *E. coli* are different from each other in chromatographic behavior, molecular weight, pH optimum and Ki value. APH(3′)-II from the *P. aeruginosa* strains and an R factor-carrying *E. coli* is apparently the same.

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