AMINOGLYCOSIDE 3′-PHOSPHOTRANSFERASE III, A NEW PHOSPHOTRANSFERASE

RESISTANCE MECHANISM

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The aminoglycoside phosphotransferase of *Pseudomonas aeruginosa* 21-75 was purified by affinity chromatography using dibekacin-Sepharose 4B or lividomycin A-Sepharose 4B followed by DEAE Sephadex A-50 chromatography. It had activities of both the known aminoglycoside 3′-phosphotransferases I and II, and transferred phosphate from ATP to the 3′-hydroxyl group of kanamycin A, ribostamycin and butirosin A and 5″-hydroxyl group of lividomycin A. This enzyme was designated aminoglycoside 3′-phosphotransferase III. It showed strong substrate inhibition by kanamycin A and ribostamycin when their concentration exceeded 6 μM. Purification and characterization of this enzyme are reported.

Aminoglycoside 3′-phosphotransferases I[1] and II[2] (kanamycin-neomycin phosphotransferases) have been reported to be involved in the resistance of clinically isolated Gram-negative bacteria, and their isoenzymes have been found in *Pseudomonas aeruginosa*[3]. However, the mechanism of resistance of a resistant strain of *P. aeruginosa* was not explained by these enzymes, and we found a new type of phosphotransferase which transfers phosphate to 3′-hydroxyl group of kanamycin, neomycin and butirosin and 5″-hydroxyl group of lividomycin. Thus, this enzyme was different from the known aminoglycoside 3′-phosphotransferases I and II in the substrate specificity and we designated this enzyme aminoglycoside 3′-phosphotransferase III.

In this paper, purification and characterization of this enzyme are reported.

Materials and Methods

Producing strain:

Pseudomonas aeruginosa 21-75 was isolated from a patient in Faculty of Medicine, Shinshu University, Matsumoto, Nagano. The growth of this strain is not inhibited by 200 μg/ml of kanamycin A, gentamicin C_1, dibekacin (3′,4′-dideoxykanamycin B), lividomycin A and ribostamycin. It is also resistant to butirosin A and amikacin [1-N-(S)-4-amino-2-hydroxybutyrylkanamycin], with minimum inhibitory concentrations of 100 μg/ml and 25 μg/ml, respectively.

Aminoglycoside antibiotics:

Kanamycin A, kanamycin B, dibekacin and ribostamycin were supplied by Meiji Seika Kaisha Ltd., lividomycin A by Kowa Co., and butirosin A by Bristol-Banyu Research Institute.

Preparation of S-100 enzyme solution:

Cells of *P. aeruginosa* 21-75 were disrupted and the supernatant (S-100) from 100,000 *g* centrifugation was prepared by the method reported previously[3].

Method of testing enzymatic activity using γ-^{32}P-ATP:

The reaction mixture (250 μl) consisted of 4~200 μM of an antibiotic, 1,600 μM ATP, 0.5 μCi of γ-^{32}P-ATP (9.24 Ci/mmole, purchased from New England Nuclear, Boston, Mass.), 25 μl of the proper buffer solution (250 or 400 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 15~120 minutes. It was then diluted with 14 ml of water and passed through a column of Amberlite CG-50 resin (NH_4^+ form, 1 ml), which was then 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) from the phosphorylated antibiotic was counted by a liquid scintillation system (Aloka LSC-653).

Affinity chromatography with dibekacin-Sepharose 4B:

Sepharose 4B with covalently bound dibekacin was prepared for affinity chromatography as described in a previous paper[1]. The column (1.5×14.0 cm) of dibekacin-Sepharose 4B was equilibrated 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. Twenty ml of the S-100 solution was applied to the column and the column was washed with 300 ml of buffer A. The enzyme adsorbed on the column was eluted with a linear gradient between 150 ml each of buffer A and buffer A containing 1.5 M sodium chloride at a flow rate of 20 ml per hour at 4°C. The eluate was cut into 4.0 ml fractions. Enzymatic activity in each fraction was determined by the method described above.

Affinity chromatography with lividomycin A-Sepharose 4B:

The active eluate (20 ml) obtained by the affinity chromatography on dibekacin-Sepharose 4B was dialyzed against buffer A and subjected to a column of (1.0×6.6 cm) lividomycin A-Sepharose 4B which was prepared by procedure similar to that for dibekacin-Sepharose 4B. The column was washed with 25 ml of buffer A, and the enzyme adsorbed on the column was eluted with a linear gradient between 50 ml each of buffer A and buffer A containing 1.5 M sodium chloride. The eluate was cut into 2-ml fractions.

Ion-exchange chromatography with DEAE-Sephadex A-50:

The active eluate (5 ml) obtained by affinity chromatography on dibekacin-Sepharose 4B was diluted 40 times with buffer A, and applied to a column of (1.6×5.0 cm) DEAE-Sephadex A-50 equilibrated with buffer A. After washing with 50 ml of buffer A, the enzyme adsorbed on the column was eluted with a linear gradient between 50 ml each of buffer A and buffer A containing 1.0 M sodium chloride. The eluate was cut into 3-ml fractions.

Disc-electrophoresis with polyacrylamide-gel:

The active eluate (5 ml) of the dibekacin-Sepharose 4B affinity chromatography was concentrated to 0.2 ml by ultrafiltration using Minicon B-15 (purchased from Amicon Co., Lexington, Mass.). To this concentrate 0.4 ml of 40 % sucrose solution was added, and 0.15 ml of the mixture was applied to each tube for gel-electrophoresis on 7.5 % polyacrylamide (2.5 mA for 90 minutes).

Estimation of molecular weight by gel-filtration:

The purified enzyme solution (5.0 ml) prepared by dibekacin-Sepharose 4B affinity chromatography was concentrated to 0.5 ml by ultrafiltration. The concentrate was applied to a column (1.5×54.0 cm) of Sephadex G-100 equilibrated with buffer A. The enzyme was eluted with buffer A at a flow rate of 20 ml per hour at 40°C. Column effluents were collected in 2-ml fractions. The molecular weight of the enzyme was estimated using standard proteins such as cytochrome C (mol. wt. 12,500), chymotrypsinogen A (25,000), ovalbumin (45,000), bovine serum albumin (67,000) and ferritin (540,000). The solution of these standard proteins was determined by measurement of the absorption at 280 nm.

Optimal pH:

The reaction mixture described in a previous section was adjusted to a specific pH by the following buffers: acetate buffer (pH 4.8~6.5), phosphate buffer (pH 6.4~7.7) and tris-hydrochloric acid buffer (pH 7.0~8.8). The effect of pH on the enzyme reaction was determined by the method described above. The enzyme solution purified by dibekacin-Sepharose 4B affinity chromatography was employed.

MICHAELIS constant (*Km*):

Km values for lividomycin A and butirosin A were obtained from LINEWEAVER-BURK plots. An enzyme solution purified by dibekacin-Sepharose 4B affinity chromatography was used and the enzymatic activity was determined by the method described above. As described in the section on Results, due to the substrate inhibition *Km* values for kanamycin A and ribostamycin could not be determined.

Inhibition constant (*Ki*):

Ki value of dibekacin against butirosin A as the substrate was obtained from LINEWEAVER-BURK plots of the data from a reaction mixture containing 15~60 μM of butirosin A and 10~40 μM of dibekacin. *Ki* value of dibekacin against lividomycin A as the substrate was obtained by DIXON plots of the data from a reaction mixture containing 30~80 μM of lividomycin A and 40~80 μM of dibekacin.

Results

Phosphorylation of Kanamycin A, Ribostamycin, Butirosin A and Lividomycin A by S-100 Enzyme Solution

Each antibiotic was added at 50 μM concentration to the reaction mixture (1.0 ml) containing 100 mM tris-hydrochloric acid buffer, pH 7.8 (or 100 mM phosphate buffer, pH 7.8), 10 mM magnesium acetate, 60 mM potassium chloride, 4 mM ATP, 10 mM dithiothreitol and 0.4 ml of S-100, and the reaction mixture was kept at 37°C for 1 hour. The reaction was stopped by heating in a boiling water bath for 3 minutes, and the antibacterial activity of the residual antibiotic was determined by a disc-plate method. Under these conditions, kanamycin A and butirosin A were not inactivated, however, lividomycin A was significantly inactivated, and ribostamycin was almost completely inactivated. The inactivated ribostamycin was isolated by the method described previously[2] and identified as ribostamycin 3′-phosphate. The inactivated lividomycin A was extracted and identified as the 5″-phosphate by comparison with an authentic sample[1]. The degree of inactivation of ribostamycin was different in the reaction mixtures containing tris-hydrochloric acid buffer or phosphate buffer, and in the latter the antibiotic was more strongly inactivated. However, lividomycin A was inactivated similarly in both reaction mixtures.

Inactivation of kanamycins A and B was studied in the reaction mixture containing two-fold diluted enzyme solution and tris-hydrochloric acid buffer or phosphate buffer, but significant inactivation of kanamycin A did not occur except for one case where S-100 was diluted two-fold and phosphate buffer was employed. However, kanamycin B was almost completely inactivated when S-100 was diluted 2~4 times and phosphate buffer was employed. Its inactivation was significantly slighter in a reaction mixture containing tris-hydrochloric acid buffer than in that containing phosphate buffer. The inactivated kanamycin B was extracted and purified by the method descirbed previously[4] and was identified as the 3′-phosphate.

Purification of the Enzyme

The S-100 solution was subjected to dibekacin-Sepharose 4B affinity chromatography, and the activity of each fraction to phosphorylate ribostamycin, lividomycin A and butirosin A was determined. As shown in Fig. 1, all the activities appeared in one peak (fractions 54~66) which was eluted with 0.5~0.7 M sodium chloride.

The active fractions thus obtained were combined and subjected to lividomycin A-Sepharose 4B affinity chromatography. Then, as shown in Fig. 2, all activities appeared in the same fractions (fractions 14~19) eluted with 0.2~0.3 M sodium chloride, producing a single peak.

The active fractions obtained by dibekacin-Sepharose 4B affinity chromatography were

Fig. 1. Dibekacin-Sepharose 4B affinity chromatography
A: Phosphorylation of ribostamycin during 15-minute incubation
B: Phosphorylation of lividomycin A during 45-minute incubation
C: Phosphorylation of butirosin A during 45-minute incubation
Each antibiotic was added at 10 μM concentration to the reaction mixture (250 μl)

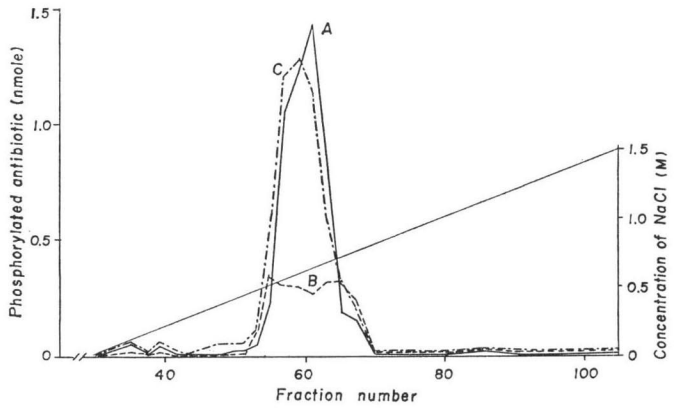


Fig. 2. Lividomycin A-Sepharose 4B affinity chromatography
A: Phosphorylation of ribostamycin during 15-minute incubation
B: Phosphorylation of lividomycin A during 45-minute incubation
C: Phosphorylation of butirosin A during 45-minute incubation
Each antibiotic was added at 10 μM concentration to the reaction mixture (250 μl)

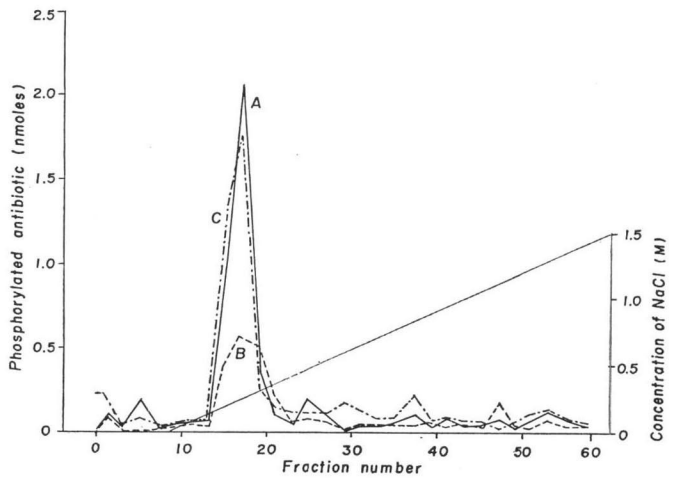
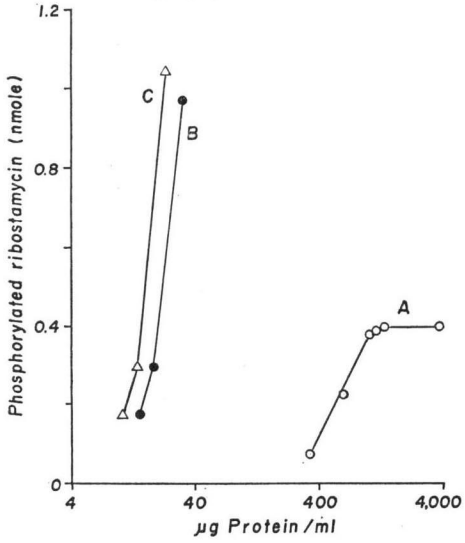


Fig. 3. Phosphorylation of ribostamycin by crude and purified enzyme preparations
A: S-100
B: Purified by dibekacin-Sepharose 4B affinity chromatography
C: Purified by lividomycin A-Sepharose 4B affinity chromatography



purified by DEAE-Sephadex A-50 chromatography and in this case also, activities for phosphorylation of ribostamycin, lividomycin A and butirosin A formed a single peak eluted with 0.1~0.2 M sodium chloride. The enzyme thus purified was very unstable and lost activity almost completely after 24 hours at 0°C or −20°C.

The rate of ribostamycin phosphorylating activity to the protein content was determined on the enzyme solutions obtained by dibekacin-Sepharose 4B affinity chromatography and lividomycin A-Sepharose 4B affinity chromatography with the results shown in Fig. 3. Though the reason is not clear, the slope of the activity *versus* protein curve was the same with the enzymes obtained by the affinity chromatography described above, but the slope was different from that obtained with S-100. Thus, the degree of purification from S-100 could not be calculated, but the curves in Fig. 3 indicate that affinity chromatography is a useful method for purification of this enzyme.

Kinetic Studies

The enzyme solution obtained by dibekacin-Sepharose 4B affinity chromatography phosphorylated kanamycin A, but as shown in Fig. 4 a strong substrate inhibition was observed at concentrations higher than 6 μM. Substrate inhibition is the reason for negative inactivation by S-100 enzyme solution. As shown in the same figure, in the reaction mixture containing tris-hydrochloric acid buffer, ribostamycin also showed substrare inhibition, though much weaker than that of kanamycin A.

In the same reaction conditions, as shown in Fig. 5, lividomycin A showed no substrate inhibition at a concentration not higher than 60 μM and its *Km* obtained from LINEWEAVER-BURK plots of the data in this range was 550 μM. Substrate inhibition was observed when the concentration of lividomycin A in the reaction mixture exceeded 100 μM. The same thing was observed also when lividomycin A-phosphorylation was studied in the reaction mixture containing phosphate buffer. In the reaction mixture containing phosphate buffer, butirosin A showed no substrate inhibition, and the *Km* value was 54 μM. In the reaction mixture containing phosphate buffer, kanamycin A and ribostamycin also showed substrate inhibition, but the degree of inhibition was weaker than in the reaction mixture containing tris-hydrochloric acid buffer.

Dibekacin with no 3′-hydroxyl group did not undergo the enzyme reaction, but showed inhibition of this enzyme reaction. Phosphorylation of butirosin A was competitively inhibited

Fig. 4. LINEWEAVER-BURK plots of phosphorylation of ribostamycin and kanamycin A by aminoglycoside 3′-phosphotransferase III in the reaction mixture containing tris-hydrochloric acid buffer, pH 7.8

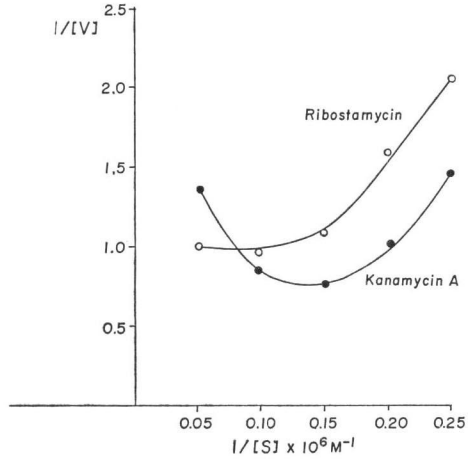


Fig. 5. LINEWEAVER-BURK plots of phosphorylation of lividomycin A and kanamycin A by aminoglycoside 3′-phosphotransferase III in the reaction mixture containing tris-hydrochloric acid buffer

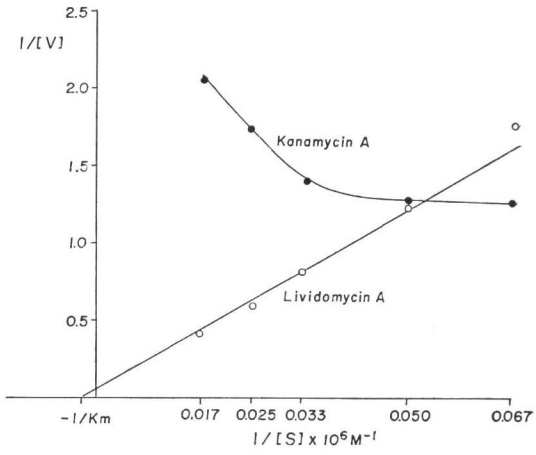
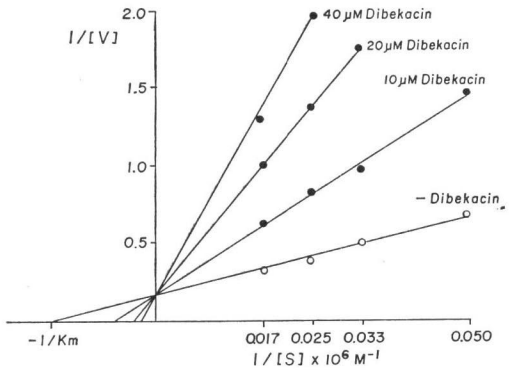


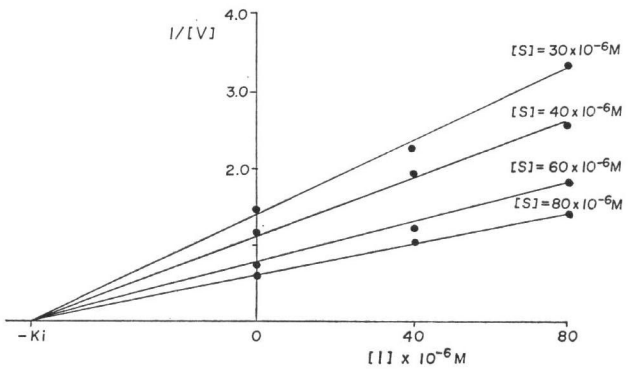
Fig. 6. LINEWEAVER-BURK plots of phosphorylation of butirosin A by aminoglycoside 3′-phosphotransferase III in the presence or absence of dibekacin.



as shown in Fig. 6 and the *Ki* was 5.67 μM. It is interesting that inhibition of phosphorylation of lividomycin A at concentrations of 30~80 μM by dibekacin is noncompetitive and the *Ki* obtained by DIXON plot of the data (Fig. 7) is 60.1 μM.

Generally, *Ki* values of an inhibitor should be independent of substrate. However, in the case of this enzyme, *Ki* value against butirosin A was significantly different from that against lividomycin A. This difference is discussed in the next section.

Fig. 7. DIXON plots of phosphorylation of lividomycin A by aminoglycoside 3′-phosphotransferase III against the concentration of dibekacin.



Characterization of the Enzyme

As described above, this enzyme phosphorylates kanamycin A, ribostamycin, lividomycin A and butirosin A, and undergoes substrate inhibition by kanamycin A and ribostamycin but not by lividomycin A and butirosin A. Using the enzyme obtained by dibekacin-Sepharose 4B affinity chromatography, substrate specificity was studied. The degree of phosphorylation of each antibiotic was examined in a reaction mixture containing 10 μM of each antibiotic and tris-hydrochloric acid buffer. As shown in Table 1, kanamycin A, kanamycin B, ribostamycin, neamine, neomycin and paromomycin were phosphorylated rapidly. Lividomycin A and butirosin A were moderately phosphorylated. Kanamycin C, paromamine, amikacin (1-N-[(S)-4-amino-2-hydroxybutyryl] kanamycin A) and 4′-deoxykanamycin were hardly reacted.

Table 1. Substrate specificity for aminoglycoside 3′-phosphotransferase III.

	Phosphorylated product (nmole)
Kanamycin A	1.03
Kanamycin B	1.48
Kanamycin C	0.10
Neamine	1.94
Paromamine	0.00
Ribostamycin	1.86
Lividomycin A	0.40
Neomycin	1.62
Paromomycin	1.39
Amikacin	0.07
Butirosin A	0.23
4′-Deoxykanamycin	0.01

Reaction was carried out for 15 minutes in the reaction mixture described in the text.

The optimal pH for the reaction determined with the enzyme obtained by dibekacin-Sepharose 4B affinity chromatography was as follows: pH 7.2 against butirosin A, pH 5.5～8.0 against lividomycin A.

The molecular weight was estimated by gel-filtration to be 25,500.

The enzyme preparation obtained by dibekacin-Sepharose 4B affinity chromatography is very unstable and a significant decrease (about 40 %) in activity was observed when it was kept for 5 minutes at 37～47°C and a more marked decrease (about 60 %) was observed at 52～62°C. The decrease of activity in phosphorylating lividomycin A or butirosin A was parallel.

Discussion

Elution patterns in affinity chromatography using dibekacin-Sepharose 4B and lividomycin A-Sepharose 4B or in DEAE-Sephadex A-50 chromatography suggest that a single enzyme obtained from *P. aeruginosa* 21-75 has the activity of both aminoglycoside 3′-phosphotransferases I and II. As previously reported[1], aminoglycoside 3′-phosphotransferase I transfers phosphate from ATP to the 3′-hydroxyl group of kanamycins, neomycins, ribostamycin and to the 5″-hydroxyl group of lividomycins, but not to the 3′-hydroxyl group of butirosins, and aminoglycoside 3′-phosphotransferase II transfers phosphate to the 3′-hydroxyl group of kanamycins, neomycins, ribostamycin and butirosins, but not to the 5″-hydroxyl group of lividomycins. The active fraction which was obtained in a single peak by chromatography of *P. aeruginosa* 21-75 enzyme phosphorylated both the 3′-hydroxyl group of kanamycins, ribostamycin, butirosin A and the 5″-hydroxyl group of lividomycin A. As described in a previous paper[3], we found a strain of *P. aeruginosa* which formed both phosphotransferases I and II, but these enzymes were separated by affinity chromatography. The substrate specificity of the enzyme of *P. aeruginosa* 21-75 in detail was not completely the same as that for a mixture of phosphotransferases I and II. For instance, paromamine which was easily phosphorylated by

phosphotransferases I and II and 4′-deoxykanamycin which underwent phosphorylation by phosphotransferase I were resistant to the action of the enzyme of *P. aeruginosa* 21-75. The enzyme of *P. aeruginosa* 21-75 was very unstable and 40~60 % decrease in the activity was observed when kept for 5 minutes at 37~62°C. In this case, the decrease in activity to phosphorylate lividomycin A was parallel with the decrease in activity to phosphorylate butirosin A. Though the enzyme of *P. aeruginosa* 21-75 was not stable enough for further purification, we could confirm that an extract from a single protein band in gel-electrophoresis phosphorylates both butirosin A and lividomycin A. Thus, it can be concluded that the enzyme obtained from *P. aeruginosa* 21-75 is a new type of aminoglycoside 3′-phoshotransferase. This enzyme was designated aminoglycoside 3′-phosphotransferase III.

Another characteristic of this enzyme is to undergo a strong substrate inhibition by kanamycin A and ribostamycin and weak substrate inhibition by lividomycin A but not by butirosin A. As described previously[3], phosphotransferase I underwent substrate inhibition when the concentration of kanamycin A, ribostamycin, or lividomycin A was 100 μM, and no substrate inhibition of phosphotransferase II was caused by 400 μM substrate antibiotics. As described in this paper, in the case of phosphotransferase III, substrate inhibition occurred when the concentration of kanamycin A or ribostamycin exceeded 6.0 μM, and substrate inhibition was significantly stronger in tris-hydrochloric acid buffer than in phosphate buffer.

A weak substrate inhibition was observed when the concentration of lividomycin A exceeded 100 μM.

Five types of mechanism of substrate inhibition have been described by WEBB[5]. Among the mechanisms described, the possibility of type-A is excluded, because the intercepts of the extension of the linear part of the curves (obtained from the slope at lower substrate concentrations) on the $1/V$ axis are in the minus region. The type-B mechanism is suggested by the kinetic data on kanamycin A and ribostamycin substrate inhibition. According to WEBB, in type-B inhibition, the substrate combines not only at the active substrate site but also at sites in various spatial relationships to the active site, interfering with either the binding of the substrate in an active enzyme-substrate-complex or interfering with its reaction when so bound. Thus, the presence of two binding sites for these antibiotics in phosphotransferase III can be imagined: the one for catalysis and another for substrate inhibition. Comparing with structures of antibiotics with strong, weak or no substrate inhibition, the 1-amino group and the 6′-amino group are suggested to be involved in the mechanism for substrate inhibition. Kanamycin A showed stronger substrate inhibition than ribostamycin (Fig. 4). It suggests that a 3-amino-3-deoxy-D-glucose moiety would enhance the inhibition. Kanamycin B was inactivated by S-100 enzyme solution under conditions where kanamycin A was not significantly inactivated. It suggests that the 2′-amino group might be lower the activity for substrate inhibition. Therefore, if there is a binding site for substrate inhibition in the enzyme, then free 1-amino and 6′-amino groups are suggested to be involved in this binding, and this binding or a conformational change caused by the binding is suggested to be enhanced by 3-amino-3-deoxy-D-glucose moiety or to be weakened by 2′-amino group.

Km values for butirosin A and lividomycin A which showed no or weak substrate inhibition were determined to be 54 μM and 550 μM, indicating a stronger affinity of the catalytic site for the former. The inhibition by dibekacin was competitive against the former and noncompetitive against the latter. From the structure, dibekacin can be imagined not only to bind with the catalytic site but also with the substrate inhibition site. When studying inhibition of butirosin A phosphorylation, 10~40 μM dibekacin was added, and for testing the effect on lividomycin A phosphorylation, the concentrations of dibekacin added were 40 and 80 μM. As discussed above, if dibekacin binds also with the substrate inhibition site and causes a conformational change of the enzyme, this change caused by such high concentrations of dibekacin may influence more strongly the phosphorylation of lividomycin A which has a lower affinity to the catalytic site than butirosin A and has a larger molecular size.

The *Ki* value of dibekacin against the phosphorylation of lividomycin A was about 10 times larger than that against the phosphorylation of butirosin A. The possibility of contamination with phosphotransferases I and II was excluded completely by careful studies on negative phosphorylation of paromamine by phosphotransferase III. It is possible that the conformation of the enzyme in the state binding to lividomycin A is different from that binding to butirosin A and the affinity of dibekacin differs with these different conformations. As described above, binding of dibekacin may also cause a conformational change of the enzyme. Therefore, due to the conformational differences, it is possible that the *Ki* values of dibekacin are different against the phosphorylations of lividomycin A and butirosin A.

In the cases of kanamycin A and ribostamycin which showed a strong substrate inhibition, optimal pH values could not be obtained. Optimal pH for lividomycin A was broad, pH 5.5~8.0, and that for butirosin A was in a narrow range, showing a sharp peak at pH 7.2. The fact that we could not obtain an optimal pH for kanamycin A and ribostamycin may be related to substrate inhibition which causes a conformational change of the enzyme and is influenced by the kind of buffer in the reaction mixture.

The molecular weight of phosphotransferase III was shown to be 25,500. This value is close to that of phosphotransferase I (27,000) and phosphotransferase II (27,000).

It is noteworthy that, due to strong substrate inhibition, if kanamycin A is added at 50 μM concentration to S-100 solution, no significant inactivation is observed. This observation indicates that if inactivation is tested for by a decrease of antibacterial activity, the presence of such an enzyme may be overlooked. In addition the strong substrate inhibition observed in this paper suggests the possible presence of a binding protein for aminoglycoside antibiotics which may be involved in the resistance mechanism.

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