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Purification and Characterization of Aminoglycoside 3′-Phosphotransferase Type IIa and Kinetic Comparison with a New Mutant Enzyme

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Aminoglycoside 3′-phosphotransferases [APH(3′)s] provide an important means for high-level resistance to neomycin- and kanamycin-type aminoglycoside antibiotics. A four-step purification which affords milligram quantities of homogeneous APH(3′) type IIa [APH(3′)-IIa] is described. The kinetic parameters for the turnover of five substrates by the enzyme were determined, and the pH dependence and metal activation for catalysis were investigated. All five cysteines in the amino acid sequence of the enzyme exist in their reduced forms; hence, there are no disulfide bonds in the protein. Modification of the cysteine thiols by S-cyanylation showed essentially no effect on the enzymatic activity. A mutant enzyme derived from APH-3′-IIa, which possesses a conservative Glu-182–Asp point mutation and which provides diminished resistance to G418 (R. L. Yenofsky, M. Fine, and J. W. Pellow, Proc. Natl. Acad. Sci. USA 87:3435–3439, 1990), was also purified to homogeneity. Kinetic analysis of this mutant protein indicated an increase of approximately ninefold in the K_m for Mg^{2+} ATP. Insofar as K_m may approximate K_s, this finding argues for the involvement of residue 182 in the binding of Mg^{2+} ATP. Thus, purified APH(3′)-IIa and a point mutant derivative enzyme were characterized enzymologically, and the roles of metal cofactors and the five reduced cysteine residues were probed in the wild-type enzyme.

Among the enzymes that modify aminoglycoside antibiotics, aminoglycoside 3′-phosphotransferases [i.e., APH(3′)s] are commonly found in resistant bacteria (4, 18, 27, 32). APH(3′)s catalyze the transfer of the γ-phosphoryl group of ATP to the 3′-hydroxyl group of kanamycins, neomycins, paromomycins, neamine, paromamine, ribostamycin, butirosin, and G418. Seven isozymes of APH(3′) have been reported in the literature to date (for reviews, see references 4, 18, 28, and 31).

In our effort toward a systematic structural and mechanistic analysis of the bacterial aminoglycoside phosphotransferases, we developed a practical purification for APH-3′ type IIa [APH(3′)-IIa] which affords several milligrams of homogeneous enzyme. Furthermore, we report here on the basic characterization of this enzyme. In addition, we report on the kinetic behavior of a mutant derivative of APH(3′)-IIa with a conservative Glu-182–Asp substitution, which conferred a lower level of aminoglycoside resistance in vivo (33).

MATERIALS AND METHODS

Molecular mass markers and the Bio-Rad protein assay dye reagent concentrate were purchased from Bio-Rad. Kanamycin, neomycin, G418, amikacin, 5,5′-dithiobis(2-nitrobenzoic acid (DTNB), ATP, ADP, phosphoenolpyruvate (PEP), NADH, *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and piperazine-*N*,*N*′-bis(2-ethanesulfonic acid (PIPES) were obtained from the Sigma Chemical Co. The cyanylating reagent 2-nitro-5-thiocyanatobenzoic acid (NTCB) was synthesized by the procedure described by Degani and Patchornik (9). Pyruvate kinase (PK) and lactate dehydrogenase (LD) were purchased from the Worthington Biochemical Co. All ultrafiltration materials were purchased from Amicon Co. [γ-^{32}P]ATP (3,000 Ci/mmol) was supplied by Dupont-NEN Research Products. Radioactivity was measured in an LS3801 Beckman liquid scintillation counter. Amino acid analysis was carried out by the method of Heinrikson and Meredith (15). Spectrofluorometric assays were performed in a Spex Fluorolog spectrometer. *Escherichia coli* HB101 (pGEME$_{182}$) and HB101(pGEMD$_{182}$), which express the wild-type APH(3′)-IIa enzyme and the Glu-182–Asp mutant enzyme, respectively, were produced by Richard Yenofsky of Phytogen (Pasadena, Calif.) by cloning the respective wild-type and mutant genes from pDAE and pDAD (33) into pGEM (Promega) and transforming *E. coli* HB101 with these plasmids. High-pressure liquid chromatography (HPLC) was carried out with a Perkin-Elmer series 410 Bio LC Pump and an LC-95 UV/visible spectrophotometer detector.

Enzyme assays. Enzymatic activity was measured by either the standard radioactive assay of γ-^{32}P transfer from ATP to an aminoglycoside (24) or the continuous coupled assay described by Goldman and Northrop (14) and adapted by Perlin et al. (25).

(i) Radioactivity assay. Aminoglycoside phosphotransferase activity at all stages of purification was determined by the phosphocellulose filter assay by using kanamycin as a phosphate acceptor. Assay mixtures contained 200 mM PIPES (pH 7.0), 30 mM $MgCl_2$, 2.5 mM dithiothreitol, 170 μM kanamycin, and 2.0 mM ATP (specific activity, 18.9 Ci/mmol), and the reaction was started by the addition of enzyme solution. Incubation of the mixture was routinely carried out at room temperature. At the designated time, the reaction was terminated by the addition of trichloroacetic acid to 10%. After centrifugation, the entire supernatant was spotted onto a square of phosphocellulose paper, which was then washed three times in 0.5% phosphoric acid, dried, and counted. The

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kinetics were found to be linear within the first 45 s by this assay. For the purpose of generating the enzyme purification table, assays were carried out for only 15 s; otherwise, for routine assays during purification, a 2-min incubation time was allowed.

(ii) Coupled spectrofluorometric assay. All kinetic parameters of the purified enzymes were evaluated by a coupled spectrofluorometric assay, as discussed by Perlin et al. (25). In the course of this assay, ATP is regenerated. Therefore, neither inhibition by ADP nor a drop in the ATP concentration occurs. A standard curve of the instantaneous drop in fluorescence intensity versus the quantity of ADP (10 to 60 μM) added to assay mixtures lacking phosphotransferase was linear. A slight modification of the assay of Perlin et al. (25) was used in the experiments, as described below.

Assays were carried out in 66 mM PIPES (pH 7.0) buffer containing 11 mM magnesium acetate, 22 mM potassium acetate, 1.76 mM PEP, 0.1 mM NADH, 6.1 U of PK, and 21 U of LD at 23.5°C in a final volume of 2 ml. Kinetic constants for each substrate (aminoglycoside or ATP) were determined in the presence of 10 nM enzyme and at least $3\times K_m$ concentrations of the second substrate. The K_m values for the aminoglycosides were determined at a constant concentration of ATP (0.15 mM for the wild-type enzyme and 2.0 mM for the Glu-182–Asp mutant enzyme). The assay mixtures were equilibrated individually at room temperature for 5 min, and then the reaction was started by the addition of ATP and the reaction was allowed to progress for 3 to 4 min. The values for K_m and k_{cat} were obtained from Lineweaver-Burk plots, typically with six to seven concentrations of substrate, each recorded in triplicate.

Purification of APH(3′)-IIa. The wild-type enzyme was purified from *E. coli* HB101(pGEME$_{182}$). Cells grown in 10 liters of YT medium containing 50 μg of ampicillin per ml were harvested in the stationary phase. All purification steps were carried out at 4°C. Protein concentrations were determined by the Bio-Rad protein assay, based on the reagent of Bradford (6).

(i) Preparation of crude extract. Eighty-five grams of wet cell paste was suspended in 60 ml of 10 mM HEPES buffer (pH 8.0) containing 10% glycerol and 10.0 mM β-mercaptoethanol (buffer A). The cells in 20-ml aliquots were disrupted by sonication (12 bursts of 30 s each, with 30-s rest intervals), and the cell debris was removed by centrifugation at 6,500 × *g* for 20 min. Streptomycin sulfate (1.05 g) was slowly added to the supernatant. The solution was stirred for 20 min and was then centrifuged at 6,500 × *g* for 20 min.

(ii) Q-Sepharose fast-flow chromatography. The cleared homogenate was diluted to 100 ml with buffer A and loaded onto a Q-Sepharose fast-flow column (27.5 by 2.6 cm; Pharmacia) which was preequilibrated in buffer A. After the column was washed with buffer A, the enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in buffer A (500 ml). After completion of the gradient, the column was washed with an additional 250 ml of buffer A containing 0.5 M NaCl. Fractions showing activity were pooled and concentrated to 1.5 ml in an Amicon concentrator.

(iii) Sephadex G-100. The concentrate was applied to a Sephadex G-100 column (130 by 1.7 cm; Pharmacia), and the protein was eluted with buffer A. Fractions with enzymatic activity were combined and were made to 5.1 M in NaCl.

(iv) Phenyl-Sepharose CL-6B. The solution from step 3 was diluted with buffer A containing 5.1 M NaCl (buffer B) and was applied to a phenyl-Sepharose CL-6B column (50 by 1.5 cm; Pharmacia) equilibrated in buffer B. The column was washed with buffer B and was then eluted with 900 ml of a linear gradient of buffer B to a solution of buffer A containing 40% glycerol. The enzyme was not eluted during the gradient, but it was subsequently eluted during a wash of the column with 250 ml of buffer B containing 40% glycerol. Fractions with activity were pooled and concentrated in an Amicon concentrator (YM10 filter). The buffer was exchanged to 10 mM sodium phosphate (pH 7.0), 60 mM KCl, and 0.01 mM $CaCl_2$ (buffer C) in the same concentrator.

(v) Hydroxylapatite chromatography. The resulting concentrate was diluted 10-fold in buffer C and was loaded onto a Bio-Gel hydroxylapatite column (50 by 1.5 cm; Bio-Rad). The column was washed with buffer C, and the protein was eluted with a linear gradient of 10 to 100 mM sodium phosphate (pH 7.0; 300 ml). The fractions with phosphotransferase activity were combined and concentrated in an Amicon concentrator. The buffer was exchanged to buffer A, and the enzyme was stored at a concentration of 10^{-4} M in the presence of 25% glycerol at −78°C. The purified enzyme retained all of its activity for at least 8 months under these conditions.

The Glu-182–Asp mutant of APH(3′)-IIa in *E. coli* HB101(pGEMD$_{182}$) was also purified to homogeneity by the method described above. The activity of the mutant enzyme was unaffected over several weeks when it was stored as described above for the wild-type enzyme.

Polyacrylamide gel electrophoresis (PAGE) and isoelectric focusing. The progress of the enzyme purification was monitored by electrophoresis in a 15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS). Typically, 30 μg of the protein was loaded in each lane, and staining of the developed gel was performed with Coomassie brilliant blue (Bio-Rad). Isoelectric focusing was carried out by the method described by Suensson-Rilbe (29). A Novex precast gel (pH 3 to 10) was developed in the cathode buffer (arginine- and lysine-free base) and the anode buffer (85% phosphoric acid) for 1 h at 100 V, 1 h at 200 V, and 30 min at 500 V.

pH dependence of catalysis. The pH dependence of catalytic turnover by APH(3′)-IIa was evaluated in the pH range of 5.5 to 9.0. The conditions were similar to those described above. Kanamycin (15 μM) was used throughout as the phosphate acceptor. Since the change in pH affects the catalytic abilities of PK and LD as well, we used large quantities of these two enzymes at the extremes of pH to ensure that catalysis by APH(3′)-IIa was being monitored exclusively as the pH was varied. The quantities of PK and LD, respectively, used at various pH levels were 27 and 63 U at pH 5.5, 18.2 and 63 U at pH 6.0, 12.1 and 21 U at pH 6.5, 6.1 and 21 U at pH 7.0, 12.1 and 42 U at pH 7.5 and 8.0, and 15 and 52.5 U at pH 9.0. The following buffers were used for the corresponding pH values: sodium acetate, pH 5.5; sodium phosphate, pH 6.0 to 6.5; sodium PIPES, pH 7.0; sodium HEPES, pH 7.5 to 8.0; and sodium borate, pH 8.5 to 9.0.

Metal dependence of catalysis. We studied the effects of replacing Mg^{2+} with Zn^{2+}, Co^{2+}, Ca^{2+}, Fe^{2+}, and Mn^{2+} on the phosphotransferase activity. Because of solubility problems, the concentrations of all metals and the potassium acetate present in the assay mixtures were reduced by one-half of the original amounts (to 5.5 mM metal and to 11 mM potassium acetate). All other assay conditions remained as described before, although non-rate-limiting concentrations of LD and PK were determined again in each case for the reasons mentioned above. The respective optimum concentrations of PK and LD were 30.4 and 42 U with Zn^{2+}, 6.1 and 21 U with Co^{2+}, and 6.1 and 31.5 U with Mn^{2+}. Inhibition by Ca^{2+} was noted for PK, whereas Fe^{2+} precipitated in solution; hence, the effects of these two metals on turnover by APH(3′)-IIa could not be studied. The buffer used was 100 mM PIPES (pH

7.0). The concentration of APH(3')-IIa used in these experiments was 1.6×10^{-7} M.

Amino acid analysis. The extinction coefficient for the wild-type APH(3')-IIa was determined from amino acid analysis, which was carried out at the Macromolecular Core Facility of School of Medicine, Wayne State University. Approximately 85 μg of the purified enzyme was dialyzed against HPLC-grade water containing 0.1% trifluoroacetic acid (TFA) (eight times with 500 ml each time for 2 days). The resulting solution was transferred to a hydrolysis tube and was concentrated to dryness in vacuo. Vapor-phase hydrolysis of the protein by 6 N HCl containing 1% phenol for 90 min at 150°C was performed on a Waters Pico Tag HPLC system by the methods of Bidlingmeyer et al. (3) and Heinrikson and Meredith (15).

Protein fragmentation. Five milligrams of enzyme was titrated with 50 μM DTNB to protect the easily accessible thiols as 5-thio-2-nitrobenzoate (TNB) derivatives. The solution was then immediately passed through a Sephadex G-15 column with 50 mM NH_4HCO_3 that was supplemented with 10 mM $CaCl_2$ (pH 8.5) to remove the excess DTNB. The fractions showing A_{280} were collected and washed with the same solvent in an Amicon concentrator until no yellow color was detected in the concentrate. The concentrated enzyme was treated with 100 μg of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin overnight with gentle stirring at room temperature. Trypsin was removed by passing the solution through a Centricon 10 (3 h, 5,000 rpm). A 40-μl portion of the filtrate was applied to a C_{18}HPLC column (0.5 by 25 cm; Vydac), and the fractions were eluted with a gradient of 5 to 95% acetonitrile in 0.1% TFA. Another 40-μl portion of the sample was subsequently treated with β-mercaptoethanol (20 μl) to reduce any disulfide bonds, and the mixture was purified on the same Vydac HPLC column under conditions similar to those mentioned above.

Cyanylation of APH(3')-IIa. All glassware, polyethylene containers, and buffers used in the following experiment were treated as described by Davies et al. (8) for experiments with metal-free solutions. The glassware was treated with 1% nitric acid (12 h) and was then rinsed with 0.01% EDTA (12 h). The polyethylene containers were washed only with 0.01% EDTA (12 h). All buffers were made free of metals by several extractions with 10 mM dithizone in carbon tetrachloride.

The potential roles of the cysteines in the phosphotransferase activity of the enzyme was tested by cyanylation of all thiol groups by NTCB. The enzyme (3.4 mg) was first dialyzed five times against metal-free HEPES buffer (220 mM; pH 7.5) containing 10% glycerol. It was expected that dialysis would remove all low-affinity metals coordinated adventitiously to the protein. A portion of the "metal-free" enzyme (0.56 mg in 475 μl) was cyanylated with NTCB (25 μl of a 220 mM stock solution) at 4°C in the buffer mentioned above. Because of the relative instability of NTCB in the buffered solution, a fresh portion of NTCB was added to the reaction mixture every 3 to 4 h. After five additions of NTCB, the excess NTCB was removed by continuous washing on an Amicon concentrator (YM10 membrane); the resultant protein solution was titrated with 1.3 mM DTNB to monitor the extent of cyanylation. The enzyme was discovered to be only partially cyanylated (80%). Subsequently, the partially cyanylated enzyme was treated overnight with six additional aliquots of NTCB, as described above. Removal of small molecules from the solution afforded a protein that had 93% protected thiols. The activity of the cyanylated enzyme was monitored by the spectrofluorometric assay described above.

Importance of metals other than Mg^{2+} for the phosphotransferase activity. The native enzyme was dialyzed over 24 h at 4°C against 2 mM 1,10-phenanthroline (two times, with 320 ml each time) in metal-free 10 mM HEPES buffer (pH 7.0) containing 10% glycerol. Subsequently, the solution was dialyzed against the metal-free buffer. The activities of the native enzyme and the metal-free enzyme were assayed by the radioactive [^{32}P]ATP assay outlined above. Metal-free reagents were used in the assay cocktail. Magnesium chloride (containing <0.0001% other heavy metals) was added to the cocktail mixture last.

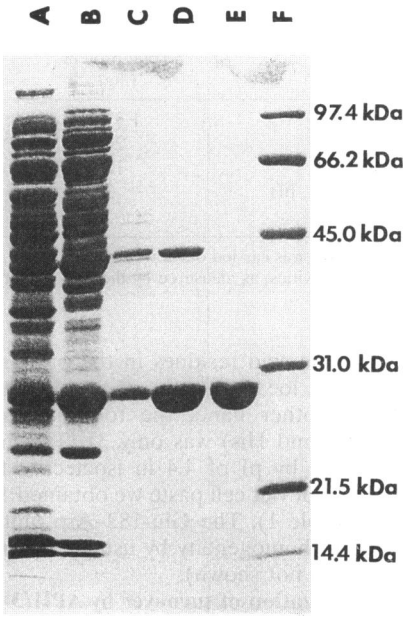

FIG. 1. SDS-PAGE of crude extract after streptomycin treatment (lane A), protein preparation after the Q-Sepharose column (lane B), protein preparation after the Sephadex G-100 column (lane C), protein preparation after the phenyl Sepharose CL-6B column, (D), protein preparation after the hydroxyapatite chromatography (lane E), and molecular size markers of (from bottom to top) 14.4, 21.5, 31, 45, 66.2, and 97.4 kDa (lane F).

RESULTS

Enzyme purification. The four-step purification of APH(3')-IIa provided a homogeneous preparation of the enzyme in milligram quantities. The Q-Sepharose ion-exchange step afforded the least purification (fivefold). However, this step eliminated a few protein bands of approximately 30 to 36 kDa that could not readily be separated from the desired protein in subsequent steps (Fig. 1). The Sephadex G-100 column affords excellent purification of the enzyme. This column separated many of the protein bands below 30 kDa. The hydrophobic interaction column, phenyl-Sepharose CL-6B, produced a highly purified protein preparation (Fig. 1), which showed only two other protein bands (45 and 66 kDa) besides the desired protein. After the hydroxylapatite column step, SDS-PAGE analysis revealed a homogeneous preparation of APH(3')-IIa, with an apparent molecular mass of approximately 30 kDa. From the primary amino acid sequence, which was deduced from the gene sequence (1), a molecular mass of 29,039 Da was calculated for the enzyme. The extinction coefficient for the protein at 280 nm was calculated as 62,900 ± 4,400 M^{-1} cm^{-1}. The protein was highly acidic. There were a total of 43

TABLE 1. Summary of the purification of APH(3')-IIa[a]

Purification step	Total protein (mg)	Total activity (nmol min^{-1} mg^{-1})[b]	Sp act (nmol min^{-1} mg^{-1})[b]	% Recovery	Purification (fold)
1. Streptomycin sulfate	1,372	21.4	1.6×10^{-2}	100	1
2. Q-Sepharose	265	21.4	8.0×10^{-2}	99.8	5
3. Sephadex G-100	107	16.2	1.5×10^{-1}	76	9
4. Phenyl-Sepharose CL-6B	30	16.0	5.4×10^{-1}	75	34
5. Hydroxylapatite	16	14.0	8.6×10^{-1}	65	54

[a] The radioactivity assay was carried out for 15 s.
[b] The error in these activities, as measured by the radioactivity assay, was relatively high.

aspartic and glutamic acid residues in the primary structure, most of which were located in the C-terminal third of the sequence. On the other hand, the total number of basic residues (Arg, Lys, and His) was only 31. This highly acidic nature was reflected by pI of 4.4 in isoelectric focusing. In summary, from 85 g of wet cell paste we obtained 16 mg of the desired protein (Table 1). The Glu-182–Asp mutant enzyme was also purified to homogeneity by using the same purification procedure (data not shown).

Kinetic characterization of turnover by APH(3')-IIa. Table 2 summarizes the kinetic parameters for turnover of five aminoglycosides. We chose aminoglycosides consisting of two rings (neamine), three rings (kanamycin, amikacin, and G418), and four rings (neomycin) for these experiments. The K_m values for the aminoglycosides, with the exception of amikacin, were typically in the lower micromolar range (1 to 15 μM) with the wild-type enzyme. The differences in the k_{cat} values were not very large from one substrate to another. In terms of the k_{cat}/K_m ratios, kanamycin, neomycin, and neamine appeared to be very favorable substrates for APH(3')-IIa, whereas G418 and amikacin were inefficient substrates. These observations are in agreement with the previous report of a high K_m and a low catalytic efficiency of APH(3')-IIa with amikacin (24).

The kinetic values for the turnover by the Glu-182–Asp mutant enzyme appeared to be remarkably similar to those of the wild-type enzyme. Indeed, with respect to $k_{cat}/K_{m(AG)}$ ratios, the mutant enzyme appeared in some cases to be slightly more active than the wild-type enzyme. The significant difference between the two enzymes appeared to be the K_m for ATP, which was approximately ninefold greater for the mutant enzyme than for the wild-type protein.

We attempted to analyze the mechanism of catalysis by the enzyme by systematically varying the concentrations of each substrate while keeping constant the concentrations of the other substrate. Analysis by construction of secondary plots was inconclusive, and no specific pattern could be discerned.

TABLE 2. Kinetic parameters for phosphorylation of aminoglycosides by the wild-type and Glu-182–Asp mutant APH(3')-IIa enzymes[a]

Substrate	Wild-type enzyme			Glu-182–Asp mutant enzyme		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} M^{-1})
Mg^{2+} ATP		45			400	
Kanamycin	4	3	1.3×10^6	3	1	3.0×10^6
Neomycin	11	6	1.8×10^6	8	1	8.0×10^6
Neamine	29	1	2.9×10^7	9	2	5.5×10^6
Geneticin	10	15	6.7×10^5	22	30	7.3×10^5
Amikacin	2	53	3.8×10^4	1	76	1.6×10^4

[a] Kinetic parameters were measured by using 66 mM PIPES (pH 7.0) at 23.5°C. Other assay conditions are given in the text. Standard deviation is ±20%.

We attributed this result to a high error rate associated with the fluorescence assay used in these experiments, which was compounded in the process of generation of secondary plots.

The pH dependence of k_{cat}/K_m for phosphorylation of kanamycin by the wild-type enzyme is shown in Fig. 2. The pH optimum and the lowest K_m value were observed at pH 7.0, which was used in the kinetic experiments. The k_{cat} values for kanamycin phosphorylation did not vary greatly (two- to threefold) over the pH range of 5.5 to 9.0.

Metal ion substitution. The phosphotransferase activity could tolerate metal substitution (Table 3). The metals Co^{2+}, Mn^{2+}, and Zn^{2+} could substitute for Mg^{2+}. We could not measure the effect of Fe^{2+} or Ca^{2+} on the APH(3')-IIa activity. We noticed that Fe^{2+} was unstable in solution, as indicated by a color change from pale green to dark yellow, accompanied by the formation of a precipitate in a time-dependent manner. It appeared that Ca^{2+} acted as an inhibitor of PK, as reported previously (2, 20).

The nature of cysteines. A number of techniques were used to shed light on the oxidation states of the five cysteines in APH(3')-IIa. We were able to titrate 4.2 thiols in the native enzyme. This measurement suggested that essentially all cys-

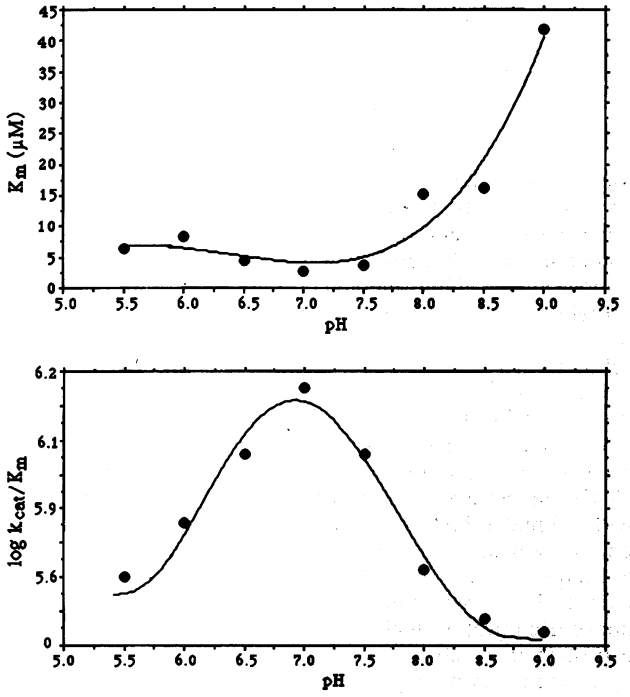

FIG. 2. pH dependence of K_m and k_{cat}/K_m for phosphorylation of kanamycin by APH(3')-IIa.

TABLE 3. Kinetic parameters for metal activation of the phosphotransferase activity for kanamycin[a]

Metal	K_m (μM)	$k_{cat}(M^{2+})/k_{cat}(Mg^{2+})$
Mg^{2+}	40	1.0
Mn^{2+}	19	0.07
Co^{2+}	16	0.07
Zn^{2+}	ND[b]	ND

[a] Kinetic parameters were measured by using 66 mM PIPES (pH 7.0) at 21.5°C. Other conditions are given in the text.
[b] ND, not determinable because of a negligible rate of reaction.

teines exist in their reduced form. We explored this possibility further by degrading the enzyme in the presence of TPCK-treated trypsin and observed the elution profile of the resultant fragments from a C_{18} reverse-phase analytical HPLC column. The elution profile did not change when the peptide mixture was treated with excess β-mercaptoethanol, suggesting the absence of any peptides containing disulfide bonds (data not shown). We wondered whether one (or more) cysteine thiols might exist coordinated to a metal in the native structure. The enzyme was titrated against 1,10-phenanthroline, a metal chelator, for removal of any potential tight-binding metal. Alternatively, the wild-type enzyme was titrated against metal-free buffer, without any added metal chelator, to remove any metals that might be coordinated adventitiously to the thiols in low-affinity binding sites. The protein preparations treated with 1,10-phenanthroline or dialyzed against metal-free buffer gave thiol titrations corresponding to 4.8 and 4.7 equivalents of cysteine thiols per protein molecule, respectively. These titrations were carried out over 20 to 40 min. The initial rates of titration were approximately 1.2-fold faster for the "demetallated" enzyme, than for the untreated wild-type enzyme.

For the wild-type and the demetallated enzymes, the cysteine thiols were more readily accessible for titration after denaturation, as judged by the fast rates of reaction with DTNB. The actual increase in the rate of titration could not be measured with accuracy because of an observed instantaneous titration of the thiols after the addition of DTNB. The method of Davies et al. (8) for the preparation of metal-free solutions and the demetallation of proteins was adhered to strictly in all of the experiments. This method is effective in removing even high-affinity metals from proteins, such as the zinc ion from carboxypeptidase A.

Effects of S-cyanylation and the metal-free state on phosphotransferase activity. We were able to cyanylate >93% of cysteine thiols. It was hoped that the introduction of the small cyano group to the cysteine thiols would not disrupt the protein structure appreciably, especially if the cysteines were located near the surface of the protein. Activity measurements by the spectrofluorometric assay of the native versus the cyanylated enzyme (3.2×10^{-7} M) showed a roughly 50% decrease in the phosphotransferase activity for the cyanylated enzyme under saturating conditions. These results suggest that the cysteine thiols do not play a critical role in the phosphotransferase activity of APH(3')-IIa. Essentially no difference in activity was noticed for the native and metal-depleted enzyme.

DISCUSSION

Because of the extensive clinical use of aminoglycosides, bacterial resistance to these agents among human pathogens has spread worldwide. A prevalent means of acquired resistance is the catalytic function of APH(3')s. Seven variants of these phosphotransferases have been identified to date (4). Understanding of the evolution of such a family of related enzymes and rational design of future aminoglycoside drugs must rely on elucidation of the structure-function relationships and the mechanistic details of such enzymes.

In this direction, we developed a practical means of purifying the prototypic member of this family of enzymes, APH(3')-IIa. Previous reports of the preparation of modest quantities of partially purified APH(3')-IIa have appeared in the literature (14, 19, 24). Each of those approaches used chromatographic columns in which aminoglycosides were incorporated as affinity column ligands. We achieved poor purification with affinity columns produced both by incorporating paromomycin and kanamycin A individually onto cyanogen bromide-activated CH-Sepharose 4B and by incorporating on Affigel the dye Cibacron blue as an affinity ligand, which typically binds to nucleotide-binding proteins. Therefore, we resorted to standard chromatographic procedures, and a successful purification sequence that afforded several milligrams of the homogeneous enzyme was developed.

With our homogeneous preparation of the APH(3')-IIa, we were able to characterize the pH profile of catalysis and analyze the effect of metal ion activation. The small effect of pH on k_{cat} indicated marginal consequences on active-site groups that are significant as acid-base catalysts. On the other hand, the K_m for kanamycin was minimal at pH 7.0, with a greater rise at a pH of >7.5 than at a pH of <6.5. One interpretation of these results is that substrate, or an active-site residue involved in binding with the substrate, undergoes titration at the basic limb of the pH profile, whereby substrate binding to the active site is impaired. The effect of protonation on substrate binding to the enzyme is much less at acidic pHs. An alternative explanation is that the two amino acids that undergo titration during catalysis—or a collection of titratable functions—are undetected by the k_{cat} measurements as a function of pH because either a crucial protein conformational change during turnover or product dissociation is rate-limiting.

Traditionally, Mg^{2+} was believed to be the metal cation of choice in phosphotransferase reactions in vivo, and indeed, this metal ion appeared to be the best metal among those tested (Table 3). The metal ions Mn^{2+} and Co^{2+} actually improved the affinity for ATP, as judged by slightly lower K_m values. However, an approximate 10-fold reduction in k_{cat} with these metals indicates that they cannot facilitate the phosphotransferase reaction as well as Mg^{2+} can. Although Zn^{2+} appeared to allow the reaction to take place, the rates were so attenuated that K_m and k_{cat} could not be evaluated accurately.

Our understanding of the structures of APH(3')s is at a rudimentary stage at present. The overall amino acid sequence homology among these proteins ranges between 24 and 42% (4, 18), with the homology increasing to 60 to 70% in the C-terminal third of the sequence (7). An analysis of conserved motifs in the protein sequence (18) and attempts at chemical mutagenesis and identification of potential important sites along the primary structure (4) have been reported. Glutamate-182 (33) and Tyr-218 (22) have been identified as possible active-site residues. A cursory inspection of the primary structures reveals that the protein sequences of the carboxy-terminal third of APH(3')s show a high number of acidic residues (i.e., Glu and Asp), suggesting that this region of the protein may interact with the amino groups of the aminoglycoside substrates. In a rather drastic attempt at testing this observation, Beck et al. (1) removed the nucleotides for the last 24 carboxy-terminal amino acids of APH(3')-IIa and showed that the resultant gene did not confer resistance to aminoglycosides. It remains to be demonstrated whether this effect is due to a lack of expression of the truncated protein, a

nonnative folding of the polypeptide, or a defective active-site topology.

Consistent with our interest in a systematic analysis of the structure and mechanism of APH(3′)-IIa, we decided to examine the roles of the five cysteine residues present in the primary structure of APH(3′)-IIa. Of these, Cys-144 was fully conserved in all APH(3′)s. While Cys-207 was present in APH(3′) types II, IV, and V, the remaining three cysteines in APH(3′)-IIa, at positions 35, 86, and 226, respectively, are not conserved among other APH(3′)s. Two questions need to be addressed when such an abundance of cysteines is noted in a protein. (i) Are there any disulfide bonds in the protein? (ii) Is there metal coordination to the cysteine thiols? An intriguing observation regarding the potential existence of metal-binding sites is the fact that the fully conserved Cys-144 is separated from His-148 by three amino acid residues. This motif is very common for metal-coordination sites in helices, where the two residues are sequestered apart from each other by one turn of the α helix; the two side chains point in the same direction and are presented as ligands to the metal (13, 16, 28, 30).

Our analysis by both thiol titration and mapping of the proteolyzed enzyme indicated the absence of any disulfide bonds in the protein. Titration of thiols was significantly accelerated in the presence of a denaturant, whereas it was relatively sluggish (20 to 40 min) in its absence. This suggests that the cysteine thiols may be sheltered inside the protein, or that strong hydrogen bonding in the protein may stabilize the cysteine thiols from reaction with the titrating agent. Furthermore, no heavy metal binding to the enzyme was apparent. Under "metal-free" conditions, the activity of the enzyme remained unaltered. In addition, modification of all five cysteines by S-cyanylation revealed only a twofold alteration in the phosphotransferase activity. These experiments suggest that the cysteine thiols are not critical for the enzymatic activity. However, they do not rule out the possibility that one (possibly Cys-144) or more cysteine thiols may serve as ligands to the Mg^{2+} in the Mg^{2+} ATP complex. If this were the case, modification of the cysteine thiols by S-cyanylation would conceivably retain the metal coordination ability in the S-cyanylated cysteine sulfide, so one would expect no profound effect on the phosphotransferase activity.

Yenofsky et al. (33) identified Glu-182 as an important residue in the resistance to aminoglycosides conferred by APH(3′)-IIa. We decided to investigate the role of this residue in the phosphotransferase activity. Insofar as K_m may approximate K_s, the ninefold increase in K_m for Mg^{2+} ATP in the Glu-182–Asp mutant enzyme suggests a lower affinity for enzyme binding to Mg^{2+} ATP. This higher K_m for the mutant enzyme may have implications of a poorer efficiency for catalysis in vivo. The intracellular concentration of ATP fluctuates at various stages of cell growth and is experimentally difficult to assess. However, for *Tetrahymena* species (11) and *Salmonella* species (5), a concentration of 3.0 to 3.3 mM has been estimated. If similar concentrations of ATP are also seen in other organisms such as *E. coli*, it should, in principle, allow the cell to overcome the lower affinity for ATP shown by the mutant enzyme. However, in light of the demonstrated lowered resistance conferred by the mutant enzyme in plants and bacteria (33), both fluctuations in ATP concentration and the location of the enzyme inside the cells should be considered. Several lines of evidence (23) point to an intracytoplasmic location of APH(3′)-IIa in *E. coli*, although a loose association with the plasma membrane has not been ruled out. Since the enzyme may be compartmentalized, one does not know the effective concentration of ATP to which it is exposed; a lower local concentration may explain the observed resistance conferred by the Glu-182–Asp mutant enzyme in vivo.

Sequence alignment of a number of phosphotransferases from bacterial, viral, and mammalian sources, including bacterial APH(3′)s, indicated that the C-terminal portions of these enzymes show some homology (7). Brenner (7) suggested that this region in these proteins is involved in a common function, namely, binding to ATP. We note that residue 182 of APH(3′)-IIa is near this region in the primary structure, which starts from residue 185. Analysis of potential secondary structural elements in the structure of APH(3′)-IIa by the method of Garnier et al. (12) revealed that residue 182 may be sequestered at the junction of a portion of the protein that shows a preference for helicity (approximately residues 140 to 180) and a section rich in β strands punctuated by β-turn regions (approximately residues 182 to 222, potentially giving rise to an antiparallel β sheet). The nature of modification in the enzyme that we studied appears to be very conservative, a replacement of a glutamic acid by aspartic acid. The only structural change is a shorter side chain for Asp by one methylene unit. The mutation resulted from a guanosine to thymidine substitution at nucleotide residue 2096 in the *neo* gene of Tn*5*. Subsequently, Yenofsky and colleagues (33) identified the presence of an identical mutation in some other commercially available plasmid vectors from different sources. We presume that the presence of this mutant has evaded detection so far because multiple copies of plasmids encoding the mutant enzyme are often present in recipient cells, so overproduction of the enzyme may overcome its catalytic deficiency. Nonetheless, the lower turnover by the mutant enyzme might have serious consequences such as a lower efficiency of selection for the *neo* marker (i.e., APH(3′)-IIa) by kanamycin or G418 in recombinant DNA experiments, as detailed by Yenofsky et al. (33).

Two potential interactions may be envisioned for the side chain of Glu-182 with Mg^{2+} ATP. First, a hydrogen bond between the Glu carboxylate and a heteroatom (of which there are several) in the ATP structure may be present. If this hydrogen bond exists, its binding must be weak (i.e., a long hydrogen bond). A second potential interaction for the side chain of Glu-182 may be as a ligand to the metal in the Mg^{2+} ATP complex. Modification of Glu to Asp at this position would remove the side chain carboxylate by a methylene unit, resulting in weaker interaction with the substrate (Mg^{2+} ATP).

We feel compelled to emphasize that a more definitive answer on the role of Glu-182 must await additional structural information. This caution is especially prudent in light of the crystal structure reported recently for the Glu-43–Asp mutant of staphylococcal nuclease (17). Those workers reported that the seemingly conservative mutation of Glu to Asp significantly perturbs the structure of the enzyme, giving rise to a less extensive network of bound water molecules in the active site. Apparently, the side chain of Asp-43 in the mutant is not long enough to reach into the active site to maintain the network of hydrogen-bonded water molecules. Otherwise, these changes caused by the mutation are considerably more drastic than would have been expected.

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