Purification, Characterization, and Investigation of the Mechanism of Aminoglycoside 3′-Phosphotransferase Type Ia[†]

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ABSTRACT: Aminoglycoside 3′-phosphotransferases [APH(3′)s] are the most common cause of bacterial high-level resistance to aminoglycoside antibiotics in clinical isolates. A one-step affinity chromatography was used to purify APH(3′) type Ia. The kinetic parameters for turnover of seven aminoglycosides and the corresponding minimum inhibitory concentrations for a strain of *Escherichia coli* harboring APH-(3′)-Ia were determined. The enzyme phosphorylates its substrates with k_{cat}/K_m values of 10^6-10^8 M^{-1} s^{-1}, including substrates such as amikacin and butirosin A which traditionally have been considered poor substrates for this enzyme. The optimal pH for the phosphotransferase activity was observed to be 7.0−7.5. The purified enzyme was found to be prone to dimerization in the absence of a reducing agent. Treatment of the enzyme with trypsin excised a 4 kDa fragment from the N-terminus which contained the amino acid residue Cys-10. The 27 kDa proteolyzed APH(3′)-Ia did not dimerize, suggesting that Cys-10 was involved in dimerization via a disulfide bond. The phophorylated kanamycin A was isolated, and the phosphorylation was confirmed to occur at the 3′-hydroxyl. Furthermore, both APH(3′)-Ia and APH(3′)-IIa were shown to phosphorylate water ("ATP hydrolase" activity) at a rate of *ca.* 10^4-10^6-fold slower (effect on k_{cat}/K_m) than that for the phosphoryl transfer to a typical aminoglycoside. The results of product-inhibition and alternative substrate diagnostics indicate an equilibrium-random mechanism for phosphorylation of aminoglycosides by APH(3′)-Ia.

Major advances in the development of novel antibiotics in the past 5 decades have been reversed by the discoveries of bacterial populations exhibiting multiple resistance to antibiotics. A state of crisis exists currently in chemotherapy of such resistant organisms (Cohen, 1992; Neu, 1992; Berkelman *et al.*, 1994), which necessitates a more intensive search for new antibiotics with novel mechanisms of action, as well as investigations geared for an understanding of the molecular bases for the resistance problem to the existing antibiotics.

Aminoglycoside antibiotics were among the first to find clinical utility (Schatz *et al.*, 1944). However, resistance of these antibacterial agents are widespread among pathogens today (Shaw *et al.*, 1993). The primary mechanism for bacterial resistance to aminoglycosides is their enzymic modifications by the family of aminoglycoside acetyltransferases, adenylyltransferases, and phosphotransferases. Among these enzyme families, aminoglycoside 3′-phosphotransferases [APH(3′)s][1] are widely represented in resistant bacteria. These enzymes, of which seven isozymes have been identified (Shaw *et al.*, 1993), catalyze transfer of the γ-phosphoryl group of ATP to the 3′-hydroxyl of many aminoglycosides such as kanamycins, neomycins, paromamine, butirosin, ribostamycin, liviodmycin, gentamicin B, geneticin, isepemicin, and amikacin (Philips & Shannon, 1984; Tieu-Cuot & Courvalin, 1986; Martin *et al.*, 1988). The phosphorylated aminoglycoside lacks antibacterial property since it no longer exhibits high affinity for binding to bacterial ribosome.

We have undertaken a systematic analysis of the family of aminoglycoside 3′-phosphotransferases, which are the most prevalent aminoglycoside-modifying enzymes in prokaryotes (Martin *et al.*, 1988). Toward that goal, we recently reported the purification and characterization of aminoglycoside 3′-phosphotransferase type IIa [APH(3′)-IIa], the best studied member of this family of enzymes (Siregar *et al.*, 1994). We describe herein purification of aminoglycoside 3′-phosphotransferase type Ia [APH(3′)-Ia] and characterization of its kinetic mechanism. APH(3′)-Ia is the enzyme in the family of aminoglycoside phosphotransferases that is found in the majority of clinical isolates and thus is an important enzyme for the resistance problem. A recent survey of 3412 clinical isolates collected from the USA, Argentina, and Belgium revealed that one-half of the isolates showed high-level kanamycin resistance associated with phosphorylation modification and that 41.6% of the kanamycin-resistant strains carried the APH(3′)-Ia gene (Shaw *et al.*, 1991). It is hoped that structural and mechanistic analyses of these enzymes would ultimately provide insight toward development of aminoglycosides which do not suffer the consequences of resistance and the syntheses of selective inhibitors for this family of enzymes.

MATERIALS AND METHODS

Kanamycin, neomycin, geneticin (G418), amikacin, lividomycin, butirosin, ATP, ADP, Nonidet P-40 (NP40), phosphoenolpyruvate (PEP), reduced form of β-nicotinamide

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[1] The three-letter notation for amino acids conforms with suggestions cited by the IUPAC-IUB Commission on Biochemical Nomenclature [(1968) *J. Biol. Chem. 243,* 3557−3559]: APH(3′), aminoglycoside 3′-phosphotransferase; PK, pyruvate kinase; LD, lactate dehydrogenase; NP40, nonidet P-40; PEP, phosphoenolpyruvate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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adenine dinucleotide (β-NADH), *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES), Sepharose CL-4B, Sephadex G-100, Sephadex G-15, cyanogen bromide, 1,8-diaminooctane, *N*-hydroxysuccinimide, *N,N*-dicyclohexylcarbodiimide, phenylmethanesulfonyl fluoride (PMSF), and trypsin were purchased from the Sigma Chemical Co. Ammonium molybdate(VI) tetrahydrate and malachite green carbinol hydrochloride were supplied by the Aldrich Co. Neamine was synthesized as described by Grapsas *et al.* (1994). Pyruvate kinase (PK) and lactate dehydrogenase (LD) were products of the Worthington Biochemical Co. The molecular weight markers, Bio-Rad protein assay dye reagent concentrate, and Coomassie brilliant blue were purchased from Bio-Rad. Ultrafiltration materials were obtained from the Amicon Co. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont-NEN Research Products.

Spectrofluorometric assays were performed in a Spex Fluorolog spectrometer. Absorbence readings were taken on a Perkin-Elmer Lambda 3B Spectrophotometer. *Escherichia coli* JM83 (pTZ18u), which expresses the wild-type APH(3')-Ia enzyme, was provided by Professor Stephen Lerner of Wayne State University Department of Medicine. Gel electrophoresis and isoelectric focusing were carried out on a Mighty Small gel electrophoresis unit (Hoefer).

^{13}C-NMR spectra were recorded on a Nicolet QE-300 spectrometer operating at 75 MHz. ^{1}H-NMR spectra were obtained on a Varian U500 spectrometer operating at 500 MHz. ^{31}P-NMR spectra were recorded on a GN-300 instrument operating at 300 MHz. An external reference of 85% H_3PO_4 in D_2O was used. Chemical shift values (δ) are given in ppm. To suppress the HOD signal, the samples were lyophilized from a solution in D_2O three times. Assignments for proton and carbon chemical shifts were based on data reported by Naganawa *et al.* (1971) and Coombe and George (1981). Mass spectra were obtained on a Kratos MS 80RFA spectrometer.

Enzyme Assays

Phosphotransferase activity was measured by either the standard radioactive assay of γ-^{32}P transfer from ATP to aminoglycoside or the continuous coupled assay described by Goldman and Northrop (1976) and adapted by Perlin *et al.* (1988).

Radioactive Assay. The phosphocellulose filter assay with kanamycin as the phosphate acceptor was used to measure aminoglycoside phosphotransferase activity for all stages of the purification and the product-inhibition kinetics by ADP. All assays were carried out in 200 mM HEPES, pH 7.5, supplemented with 30 mM $MgCl_2$ and 2.5 mM dithiothreitol. The typical assays also contained kanamycin A (170 μM) and ATP (2 mM, specific activity 31.2 Ci/mol). The Mg^{2+}-ADP product-inhibition assays were conducted at several concentrations of Mg^{2+}ADP (100−500 μM) in the presence of kanamycin A (170 μM) with variable concentrations of Mg^{2+}ATP (25−200 μM). The specific activity was calculated for each concentration of Mg^{2+}ATP.

All reactions were started by the addition of the enzyme (10 nM final). The reactions were carried out for 30 s at room temperature and then terminated by the addition of trichloroacetic acid to a final concentration of 10%. Following centrifugation, 80 μL of the supernatant was spotted onto phosphocellulose filter paper (1 × 1 cm), which was then washed with 0.5% phosphoric acid (3 × 600 mL) for 2 min, dried, and counted. The kinetics were found to be linear within the first 60 s by this assay.

Coupled Spectrofluorometric Assay. Kinetic parameters for the purified enzyme were determined by a modification of the coupled spectrofluorometric assay of Perlin *et al.* (1988), as described by Siregar *et al.* (1994). This assay involves regeneration of ATP, avoiding the possibility of inhibition by ADP or a diminution of the ATP concentration in the course of turnover. A standard plot for the instantaneous drop in fluorescence intensity versus the quantity of ADP (10−60 μM) in the absence of the phosphotransferase was linear. Standard plots were also constructed for GDP and UDP.

All assays were carried out at 23.5 °C in a final volume of 1 mL and contained 200 mM HEPES (pH 7.5), 22 mM potassium acetate, 11 mM magnesium acetate, 1.76 mM PEP, 0.1 mM NADH, 18.2 units of PK, and 21 units of LD. In determining the kinetic parameters for the nucleotides, the following quantities of PK were used for the corresponding nucleotides: with ATP, 18.2 units; with GTP, 18.2 units; and with UTP, 109 units. The steady-state kinetic parameters for turnover were determined in the presence of 10 nM enzyme and at least $3K_m$ concentrations of the second substrate (nucleotide or aminoglycoside). In the case of the steady-state kinetics of the mixture of dimeric and monomeric forms of the enzyme, 9.95×10^{-6} M protein (calculated by Bradford assay) was used. For the alternative substrate assays, the kinetic parameters for turnover of amikacin were determined at saturating concentrations of ATP, UTP, and GTP. Likewise, for determination of the kinetic parameters for ATP, saturating concentrations of kanamycin A, neomycin, and amikacin were used.

Kinetic parameters for the ATP hydrolase ("ATPase") activity of the purified enzyme in the absence of aminoglycosides were measured in the ATP concentration range of 25−500 μM in the presence of 10^{-7} M enzyme. Dibekacin (0−150 μM) inhibition kinetics were carried with 10^{-8} M enzyme and kanamycin A (10 and 20 μM). Product-inhibition assays with kanamycin A 3'-phosphate (1−50 mM) were attempted in the presence of kanamycin A (2.5 and 7.5 μM). Product-inhibition assays with Mg^{2+}ADP (0−0.5 mM) were carried out in the presence of kanamycin A (15 μM). The assay mixtures were incubated at 23.5 °C for 5 min, and then the reactions were started by the addition of the nucleotide and allowed to progress for 5 min. The values for K_m and k_{cat} were determined from Lineweaver−Burk plots, and the K_i values were obtained from both Dixon and Lineweaver−Burk plots.

Plasmid Isolation and DNA Sequencing of APH(3')-Ia. The plasmid of APH(3')-Ia was isolated using continuous cesium chloride gradient in accordance with the procedure described by Sambrook *et al.* (1989). The DNA sequence of the gene for APH(3')-Ia was determined by the Sanger dideoxy-mediated chain-termination method as described by Sanger *et al.* (1977).

Preparation of the Affinity Resin. The neomycin-coupled affinity resin was synthesized by the general method of Cuatrecasas and Parikh (1972). Well-washed Sepharose CL-4B (50 mL) was combined with an equal volume of deionized water followed by the addition of 10 g of finely powdered cyanogen bromide (200 mg/mL Sepharose), and

the reaction mixture was stirred gently. The pH of the suspension was immediately adjusted to and maintained at 11 by the addition of 8 M NaOH. The temperature was maintained at 20 °C by periodic addition of ice into the reaction mixture. The completion of the reaction was indicated by a lack of decrease in pH of the reaction mixture (10−15 min). Subsequently, the suspension was filtered and washed under suction with cold borate buffer (pH 10). The resin was resuspended in 50 mL of borate buffer (pH 10) to form a slurry, to which 1,8-diaminooctane (14.43 g, 2 mmol/mL Sepharose) was added. The reaction was allowed to continue overnight at 4 °C under mechanical shaking. The resin was then filtered and washed with deionized water to remove excess 1,8-diaminooctane followed by the addition of succinic anhydride (6.0 g, 1.2 mmol/mL Sepharose) dissolved in 30 mL of deionized water. The reaction mixture was again mixed by mechanical shaking at 4 °C. During the 5 h coupling reaction, the pH was maintained at 6 by periodic addition of 8 M NaOH. The resin was washed with dioxane (500 mL) and suspended in 100 mL of dioxane followed by the addition of 1.6 g of *N*-hydroxysuccinimide (0.014 mol) and 2.9 g *N,N*-dicyclohexylcarbodiimide (0.014 mol, final concentration 0.1 M). The mixture was allowed to stir gently at room temperature for 2 h. The activated Sepharose was then washed with cold deionized water (2 L) and resuspended in 0.1 M MOPS buffer (pH 7.5, 100 mL). Finally, neomycin (3.28 g, 3.6 mmol) was added to the activated resin followed by 4 h of incubation by mechanical shaking at 4 °C. The affinity resin was equilibrated with 25 mM HEPES, pH 7.5, for purification of APH(3′)-Ia.

Purification of APH(3′)-Ia

APH(3′)-Ia was purified from *E. coli* JM83 (pTZ18u) by a modification of the method described by Lee *et al.* (1991) for the purification of APH(3′)-Ic.

Preparation of Crude Extract. Wet-cell paste (24 g) was suspended in 30 mL of 50 mM HEPES, pH 7.5, 0.2 mM dithiothreitol, and 1 mM PMSF (buffer A). The cells were disrupted by sonication (12 bursts of 30 s each, with 30 s rest intervals), and the cell debris was removed by centrifugation (6500*g*/30 min). The supernatant was collected (32 mL), and the pellet was resuspended in 30 mL of buffer A and resonicated as mentioned above. Centrifugation (6500*g*/30 min) yielded 31 mL of supernatant which was combined with the 32 mL of supernatant from the first sonication. Streptomycin sulfate (1.32 g) was slowly added to the combined supernatant. The solution was stirred for 30 min at 4 °C followed by centrifugation at 6500*g* for 40 min. The supernatant was concentrated (14 mL) in an Amicon concentrator, and the buffer was simultaneously exchanged to 25 mM HEPES, pH 7.4, and 0.2 mM dithiothreitol (buffer B).

Affinity Chromatography. The protein concentrate was diluted up to 50 mL with buffer B and applied to the affinity column (30 × 1 cm) at a flow rate of 0.2 mL/min. The column was subsequently washed with buffer B until the absorbance of the eluent at 280 nm was <0.1. The column was then eluted with buffer B containing 0.5 M KCl (150 mL) followed by elution with the same buffer containing 1.0 M KCl (200 mL), until the absorbance at 280 nm of the eluent was approximately that of the background (<0.06). The desired protein was finally eluted with 50 mM HEPES buffer, pH 7.4, which contained 0.2 mM dithiothreitol, 1.5 M KCl, and 3 mM neomycin (buffer C). The fractions containing the phosphotransferase activity were pooled and concentrated. The buffer was exchanged to 10 mM HEPES buffer (pH 7.5), containing 1 mM DTT and 35% glycerol. Under these conditions, full activity of the stored enzyme was retained for at least 2 months at 4 °C.

Polyacrylamide Gel Electrophoresis (PAGE) and Isoelectric Focusing. Enzyme purification by affinity chromatography was monitored by electrophoresis in a 15% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate (SDS). The developed gel was stained with Coomassie brilliant blue. The amount of protein loaded in each well was 20 μg.

The Mighty Small gel electrophoresis unit was used to carry out one- and two-dimensional isoelectric focusing following the procedure described by Anderson (1991). Approximately 20 μg of enzyme was loaded on a 3.3% acrylamide tube gel (1.5 mm i.d.) which contained 2.0% ampholyte (pH 3−10; BioRad), 9.0 M urea, and 2.0% NP40 (nonionic detergent). The one-dimensional tube gel was developed for 3 h at 500 V in the presence of the cathode buffer (20 mM NaOH) and the anode buffer (0.085% phosphoric acid). The tube gel was then extracted from the tube and transferred to a two-dimensional 15% acrylamide slab gel containing 0.1% SDS. The slab gel was developed for 1.5 h at 20 mA and then stained in Coomassie brilliant blue. Protein markers within a p*I* range of 3.8−7.6 and a molecular weight range of 17−89 kDa were used as standards.

Antibiotic Susceptibility. MICs were determined in Mueller−Hinton broth according to the method of Steers *et al.* (1959). *E. coli* JM83 (pTZ18u) was grown in 5 mL of Mueller−Hinton media overnight (5×10^9 cells/mL). Subsequently, the culture was diluted to a final cell concentration of 1×10^5 cells/mL. A volume of 50 μL of this culture was added to 50 μL of Muller−Hinton broth with increasing concentrations of aminoglycosides (0−1024 μg/mL) and grown for 14 h at 37 °C. The MIC was assigned as the concentration of aminoglycoside where no visible growth was observed.

Dimerization of APH(3′)-Ia. A solution of 12 μg of APH(3′)-Ia (0.4 pmol) was stirred with $K_3Fe(CN)_6$ (13.3 μg, 40 pmol) for 2 h at 4 °C. Subsequently, the excess ferricyanide was removed, and the buffer was exchanged to 25 mM HEPES, pH 7.4, in an Amicon concentrator. Separation of the dimer and monomer was attempted by Sephadex G-100 (2 × 85 cm; Pharmacia), Sephadex G-75 superfine (2 × 57 cm), and Superose 12 (1 × 25 cm) FPLC chromatography.

Amino Acid Analysis. The extinction coefficient for APH(3′)-Ia was determined by amino acid analysis, which was carried out at the Macromolecular Core Facility, School of Medicine, Wayne State University. Approximately 26 μg of enzyme was dialyzed against double-deionized water containing 0.1% TFA (10 × 400 mL). The dialysate was then transferred to a hydrolysis tube and concentrated to dryness *in vacuo*. Vapor-phase hydrolysis of the enzyme was performed in a Waters Pico Tag HPLC system by the methods of Bidlingmeyer *et al.* (1984) and Heinrikson and Meredith (1984).

pH Dependence of Catalysis. The pH dependence of the phosphotransferase activity of APH(3′)-Ia was determined

in the pH range of 6.0−8.5. Conditions were similar to those described for the coupled spectrofluorometric assay above. The catalytic efficiencies of PK and LD are also dependent on pH; therefore, the quantities of PK and LD were adjusted at each pH to ensure that catalysis by APH(3′)-Ia was being measured exclusively as the pH was varied. The amounts of PK and LD, respectively, used at various pH levels were 36.4 and 42 units at pH 6.0 and 6.5, 18.2 and 42 units at pH 7.0, 18.2 and 21 units at pH 7.5, 24.2 and 42 units at pH 8.0, and 24.2 and 42 units at pH 8.5. The buffers used for the corresponding pH values are as follows: sodium phosphate, pH 6.0 and 6.5; PIPES-Na, pH 7.0; HEPES-Na, pH 7.5 and 8.0; and sodium borate, pH 8.5.

Detection of Inorganic Phosphate as a Consequence of the ATP Hydrolase Activity. The malachite green dye assay (Baykov *et al.*, 1988) was used to establish a correlation between the ATP hydrolase activity and the formation of inorganic phosphate. The malachite green dye stock solution was prepared by dissolving malachite green (0.44 g) into 2.5 M H_2SO_4, and the solution was stirred until it was bright orange. The final dye assay solution was prepared by combining 5 mL of 7.5% ammonium molybdate, 20 mL of the malachite green dye stock solution, and 0.4 mL of 11% Tween 20.

The enzyme (10^{-7} M) was incubated with Mg^{2+}ATP in the presence of 200 mM HEPES, pH 7.5, supplemented with 22 mM potassium acetate, and 11 mM magnesium acetate. The hydrolase activity was monitored as a function of time by periodically removing aliquots (200 μL) which were diluted into 600 μL of deionized water followed by treatment with the malachite green assay dye solution (200 μL). The solution was mixed, and the color was allowed to develop for 10 min at room temperature, at which time the absorbence at 630 nm was measured. Quantitative measurement of the inorganic phosphate released was made by the use of a standard plot of optical density at 630 nm versus known concentrations of NaH_2PO_4 (5−80 μM).

Fragmentation of APH(3′)-Ia by Trypsin. APH(3′)-Ia (4 mg/mL) was dialyzed against 100 mM Tris, pH 8.0, and 20 mM $CaCl_2$ and subsequently diluted 4-fold. The dialyzed enzyme was treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin [1:70, w/w, with respect to APH(3′)-Ia] which was dissolved in 1 mM HCl, supplemented with 2 mM $CaCl_2$, followed by incubation overnight with gentle stirring at 4 °C. Proteolysis was terminated by the addition of PMSF (500 μM). The lower molecular weight fragments (<10 kDa) were removed by Sephadex G-15 chromatography. The size of the large, major peptide fragments was determined by separation of the fragments on a Sephadex G-100 column followed by analysis on SDS−PAGE.

Preparation of the peptide fragments for peptide sequencing was carried out by Western blotting, according to the procedure provided by Bio-Rad. An SDS−PAGE of the fragmented enzyme solution (50 μg) was developed and then equilibrated in 25 mM Tris buffer (pH 8.0−8.5) containing 192 mM glycine and 25% methanol for 15−20 min (Towbin *et al.*, 1979). The transfer of the peptide fragments from the SDS gel to the Immobilon poly(vinylidene difluoride) (PVDF) transfer membrane (Millipore) was carried out on a Bio-Rad trans-blot cell as described by Bio-Rad (40 min at 200 mA). The membrane was then treated with Amido Black followed by destaining with 30% 2-propanol and 7% acetic acid. After the membrane was air-dried, the 27 kDa peptide band was cut out for peptide sequencing.

Isolation of Kanamycin A 3′-Phosphate. E. coli JM83 (pTZ18u) was grown in 5 L of the YT medium containing 50 μg/mL ampicillin and was harvested in the stationary phase. The cell paste was suspended and sonicated as described for the enzyme purification procedure. After treatment with streptomycin sulfate (1.05 g), the crude extract was centrifuged and the supernatant was dialyzed against 10 mM HEPES, pH 7.5, and 1 mM dithiothreitol (2 × 1.5 L) to remove small molecules. The dialysate was used in the following phosphorylation reaction.

The preparative phosphorylation reaction of kanamycin A consisted of the dialysate (which contained the crude enzyme preparation), 200 mM HEPES, 11 mM magnesium acetate, 1 mM dithiothreitol, and 22 mM potassium acetate in a volume of 250 mL of deionized water. The pH of the reaction mixture was then adjusted to 7.5 with 6 M NaOH. To start the reaction, kanamycin A (100 mg), PEP (40.6 mg), PK (31.5 units), and ATP (0.51 g) were added consecutively to the mixture and the reaction was incubated at 37 °C. The same quantities of kanamycin A, PEP, PK, and ATP were added again at 4 and 8 h of the reaction. The phosphorylation reaction was terminated after 12 h by placing the reaction mixture in boiling water for 10 min. The mixture was brought to room temperature and filtered (Whatman No. 1 filter paper) by suction to remove the precipitated proteins. The yellow filtration was loaded onto an IRC-50 (Na^+ form) ion-exchange column (30 × 1 cm) at a flow rate of 0.5 mL/min. The column was washed with 500 mL of deionized water followed by 1% NH_4OH at a flow rate of 1.4 mL/min. The fractions giving a positive ninhydrin test were combined and concentrated *in vacuo* to a brown oily liquid, which was then dissolved in 10 mL of deionized water and loaded onto a GC-50 (NH_4^+ form) column (30 × 1 cm). After washing with deionized water (150 mL), the column was eluted with 0.25% NH_4OH at a flow rate of 1.1 mL/min. The fractions with a positive ninhydrin test were combined and concentrated to dryness *in vacuo*.

The progress of the phosphorylation reaction was monitored by silica gel TLC (EtOH/MeOH/NH_4OH/H_2O, 5:5:4.5:4.5). Before each addition of kanamycin A, analysis of the reaction mixture by TLC showed a single spot with an R_f value of 0.72, as compared to the kanamycin R_f value of 0.38. The single spot was analyzed by various methods and identified to be kanamycin A 3′-phosphate (170 mg): yield, 56%; mp 260−280 °C dec; ^{31}P-NMR (D_2O) δ 4.52; ^{13}C-NMR (D_2O, pH 9.5) δ 32.5 (C_2), 40.4 ($C_{6'}$), 48.5 (C_1), 50.4 (C_3), 54.6 ($C_{3''}$), 60.0 ($C_{6''}$), 67.5 ($C_{4''}$), 68.7 ($C_{4'}$), 70.1 ($C_{5''}$), 71.1 ($C_{2''}$), 72.2 ($C_{5'}$), 73.4 (C_5), 75.4 ($C_{2'}$), 75.5 ($C_{3'}$), 82.6 (C_6), 97.0 ($C_{1'}$), 100.1 ($C_{1''}$); MS FAB$^+$ 565 (M + H, 1.3).

RESULTS

DNA sequencing of the APH(3′)-I resistance gene cloned into pTZ18u and expressed in *E. coli* JM83 indicated that the gene encoding for APH(3′)-Ia from Tn903 was present. The one-step purification by the neomycin-conjugated affinity chromatography yielded an essentially homogeneous protein of *ca.* 31 kDa (Figure 1). The unbound protein, as well as protein which was eluted with buffer B supplemented with 0.5 M and 1.0 M KCl, exhibited <1% phosphotransferase activity when compared to the protein eluted with

Table 1: Table for Purification of APH(3′)-Ia

purification step	total protein (mg)	total activity (nmol min^{-1})	specific activity (nmol min^{-1} mg^{-1})	recovery (%)	purification (-fold)
1. crude extract	1313	0.21	1.6×10^{-4}	100	1
2. streptomycin sulfate	737	0.14	1.9×10^{-4}	67	1.2
3. neomycin-conjugated affinity column	5	0.07	1.5×10^{-2}	33	97

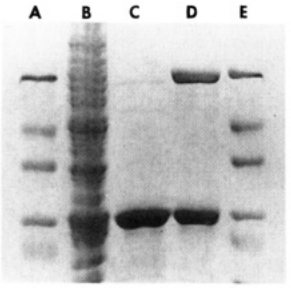

FIGURE 1: SDS−PAGE of purification of APH(3′)-Ia. Lanes A and E are molecular weight markers; from the top to the bottom: bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine erythrocyte carbonic anhydrase (29 kDa), and bovine pancreas trypsinogen (24 kDa). Crude extract (lane B), purified APH(3′)-Ia (lane C), and purified APH(3′)-Ia after treatment with ferricyanide (lane D) are shown. Approximately 20 μg of protein was introduced in each lane.

Table 2: Kinetic Parameters for Phosphorylation of Aminoglycosides by APH(3′)-Ia and Corresponding Minimum Inhibitory Concentrations (MIC) for *E. coli* JM83 (pTZ18u) Harboring the Enzyme

substrate	K_m (μM) KM	K_m (μM) ATP[a]	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{1})	MIC (μg/mL)
kanamycin A	1.2 ± 0.2	19.7 ± 2.5	102 ± 14	8.5×10^7	>1024
neomycin B	3.6 ± 0.3	32.6 ± 5.3	183 ± 13	5.1×10^7	1024
neamine	2.3 ± 0.4		141 ± 35	1.2×10^8	>1024
lividomycin A	6.6 ± 1.1		294 ± 48	4.5×10^7	>1024
amikacin	84 ± 44	26.6 ± 1.7	165 ± 46	2.0×10^6	8
butirosin A	40 ± 1		76 ± 1	1.9×10^6	4
geneticin (G418)	10 ± 1		158 ± 18	1.3×10^8	>1024

[a] These values were calculated at saturating concentrations of aminoglycosides ($3K_m$).

buffer containing 1.5 M KCl and 3 mM neomycin. From 24 g of wet-cell paste, 5 mg of the desired protein was obtained (Table 1). The enzyme was purified as a monomer; however, it was observed that partial dimerization of the monomer occurred over time in the absence of DTT. The dimer can be fully converted back to the monomer by the addition of DTT. Dimerization of the monomer could be facilitated by the addition of potassium ferricyanide (Figure 1). However, purification of the dimer from the monomer by gel filtration using Sephadex G-100, Sephadex G-75 superfine, and Superose 12 (FPLC) columns was unsuccessful. The extinction coefficient (ϵ_{280}) for the monomeric protein at 280 nm was calculated at 46 700 ± 730 M^{-1} cm^{-1}. The p*I* for APH(3′)-Ia was determined to be 5.1.

The steady-state kinetic parameters for turnover of seven aminoglycosides and their corresponding minimum inhibitory concentrations (MICs) for a strain of *E. coli* harboring APH(3′)-Ia are summarized in Table 2. The K_m values for the aminoglycosides, with the exception of amikacin and butirosin A, are in the 1-10 μM range. Furthermore, the k_{cat} values are generally within 3-fold of each other. The favorable substrates (kanamycin A, neomycin B, neamine, lividomycin A, geneticin) have k_{cat}/K_m values of 10^7-10^8 M^{-1} s^{-1},

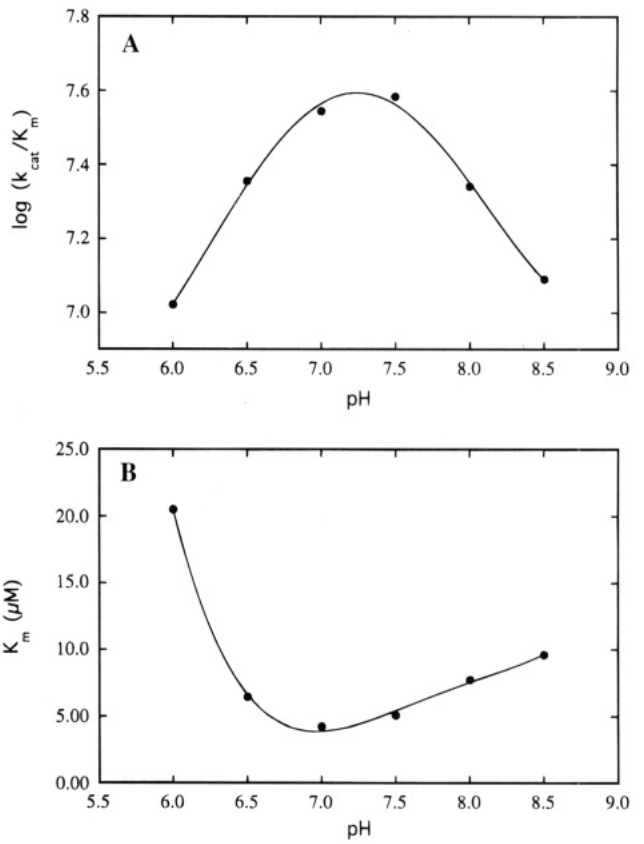

FIGURE 2: pH dependence of (A) k_{cat}/K_m and (B) K_m for turnover of kanamysin A by APH(3′)-Ia.

whereas the less favorable substrates (amikacin, butirosin A) have k_{cat}/K_m values of *ca.* 10^6 M^{-1} s^{-1}. Inhibition kinetics with dibekacin (3′,4′-dideoxykanamycin B) yielded a K_i value of 62 μM. Steady-state kinetics were carried out with a 1:1 mixture of dimer and monomer of APH(3′)-Ia. The kinetic parameters for kanamycin A (K_m = 4.7 μM, k_{cat} = 258 s^{-1}, $k_{cat}/K_m = 5.5 \times 10^7$ M^{-1} s^{-1}) and neomycin B (K_m = 6.3 μM, k_{cat} = 483 s^{-1}, $k_{cat}/K_m = 7.7 \times 10^7$ M^{-1} s^{-1}) were very similar to the results given in Table 2 for the monomeric enzyme. Hence, whether the enzyme is dimeric or monomeric has no appreciable effect on catalysis; this result is similar to that reported for APH(3′)-III (McKay *et al.*, 1994).

The pH dependence of k_{cat}/K_m for phosphorylation of kanamycin A by the wild-type enzyme is shown in Figure 2. The pH optimum for the enzyme is at *ca.* 7.0−7.5. The effect of pH on the k_{cat} values was minimal (2-fold), but an increase for K_m as observed at the pH extremes, more so at lower pH values. We were unable to accurately investigate the pH effect on the phosphotransferase activity at pH values below 6.0 and above 8.5 due to the limitations of the activity assay.

An attempt at study of the mechanism of the monomeric APH(3′)-Ia by systematically varying the concentration of one substrate while keeping constant the other substrate

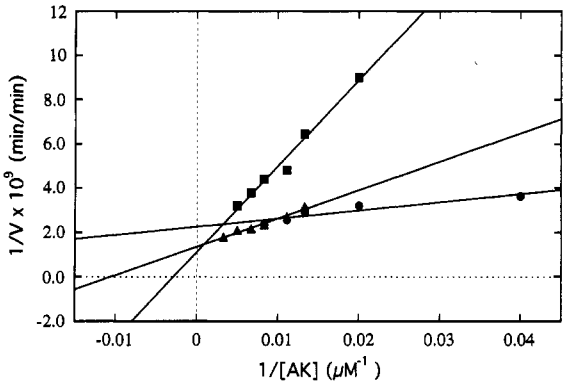

FIGURE 3: Alternative substrate analysis with amikacin as the variable-concentration substrate. The following alternative nucleotide substrates were present: Mg^{2+}ATP (200 μM) (●), Mg^{2+}GTP (400 μM) (▲), and Mg^{2+}UTP (400 μM) (■). The concentration range of amikacin was 10−120 μM for Mg^{2+} ATP, 75−300 μM for Mg^{2+}GTP, and 5−200 μM for Mg^{2+}UTP.

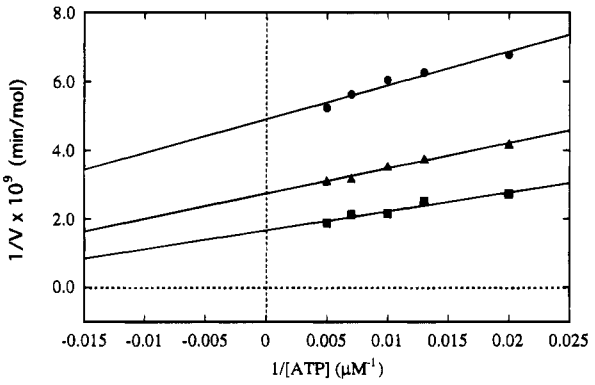

FIGURE 4: Alternative substrate analysis with Mg^{2+}ATP as the variable-concentration substrate. The following alternative aminoglycoside substrates were present: kanamycin A (24 μM) (●), neomycin B (72 μM) (■), and amikacin (300 μM) (▲). The concentration range of ATP was 50−200 μM for all three aminoglycosides.

concentration and analysis of the data by construction of secondary plots were not conclusive. Therefore, kinetic analysis by the alternative substrate method was employed. Kinetic determinations with amikacin—whose higher K_m value provides a greater slope effect—in the presence of saturating concentrations of Mg^{2+}ATP [K_m(AK) = 41 ± 9 μM, k_{cat} = 1.0 ± 0.1 s^{-1}], Mg^{2+}GTP [K_m(AK) = 94 ± 10 μM, k_{cat} = 1.7 ± 0.1], and Mg^{2+}UTP [K_m(AK) = 347 ± 103 μM, k_{cat} = 2.1 ± 0.6 s^{-1}] were carried out. The kinetic parameters for Mg^{2+}CTP and Mg^{2+}TTP could not be determined due to poor turnover of CDP and TDP by PK, an enzyme used in the coupled spectrofluorometric activity assay. Apparent values for k_{cat} and K_m varied by 2- and 8.5-fold, respectively. The resulting pattern of three intersecting lines is shown in Figure 3.

Alternative substrate kinetics with amikacin, kanamycin A, and neomycin B (K_m values were obtained with Mg^{2+}-ATP) were determined while concentrations of Mg^{2+}ATP were varied (Table 2). Comparison of the k_{cat} and K_m values with respect to the alternative aminoglycosides shows a difference of less than 2-fold and provides the plot shown in Figure 4. The lines intersect in the left quadrant.

Kanamycin A 3′-phosphate and Mg^{2+}ADP were both studied as product inhibitors. The ADP inhibition kinetics were performed by the radioactive assay, since ADP is

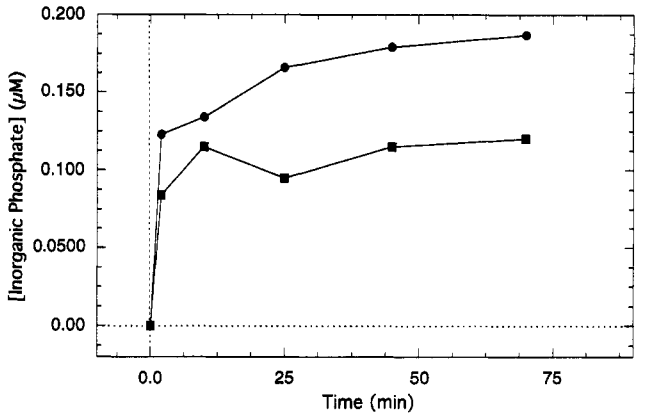

FIGURE 5: Formation of inorganic phosphate during ATP hydrolase activity of APH(3′)-Ia (●) and APH(3′)-IIa (■) as monitored by the malachite green assay at 630 nm. The concentration of ATP was 100 μM in both cases.

consumed and may not be used as an inhibitor in the spectrofluorometric assay. The results showed competitive inhibition with Mg^{2+}ADP with respect to Mg^{2+}ATP (data not shown). In the case of phosphorylated kanamycin A, product-inhibition experiments were carried out with kanamycin A 3′-phosphate concentrations up to 50 mM. Even in the presence of 50 mM kanamycin A 3′-phosphate, only a mere 20% decrease in phosphotransferase activity was observed.

Both APH(3′)-Ia and APH(3′)-IIa [purified as reported by Siregar *et al.* (1994)] showed low levels of ATP hydrolase activity. In the absence of aminoglycosides, ATP is hydrolyzed with the following kinetic parameters: APH(3′)-Ia, K_m = 49 ± 1 μM, k_{cat} = 0.023 ± 0.002 s^{-1}, k_{cat}/K_m = 6.0 × 10^2 M^{-1} s^{-1}; APH(3′)-IIa, K_m = 52 ± 19 μM, k_{cat} = 0.027 ± 0.001 s^{-1}, k_{cat}/K_m = 5.3 × 10^2 M^{-1} s^{-1}. The K_m values for ATP for the ATPase activity are in the same range as those for the phosphotransferase activity in phosphorylation of aminoglycosides. However, the value for k_{cat} is attenuated by as much as 4000-fold for the ATP hydrolase activity.

We showed independently that water was the phosphate acceptor in turnover of Mg^{2+}ATP in the absence of aminoglycosides. The formation of inorganic phosphate was documented by the malachite green assay for both APH(3′)-Ia and APH(3′)-IIa (Figure 5). An attempt to correlate the extent of inorganic phosphate formation with the formation of Mg^{2+}ADP indicated that both products formed in concert, and the measured concentrations for each product at fixed time points agreed to each other within a 5−10-fold range for both enzymes. Control experiments involving incubation of the enzyme with ADP or kanamycin or assays in the absence of enzyme or ATP showed negligible formation of inorganic phosphate (data not shown).

When incubated with TPCK-treated trypsin, APH(3′)-Ia was fragmented into three main peptide fragments corresponding to the molecular weights 27, 17, and 13 kDa. The intact protein at 31 kDa was also present. Peptide sequencing of the 27 kDa fragment revealed an N-terminal sequence of Leu-Tyr-Gly-Lys-Pro-Asp corresponding to a cleavage site at the junction of Arg-42 and Leu-43 peptide linkage. The 4 kDa N-terminal fragment (residues Met-1−Arg-42) contains a cysteine residue (Cys-10). Attempts to dimerize the 27 kDa fragment in the presence of $K_3Fe(CN)_6$, as in the case of the 31 kDa monomer, did not provide any dimeric protein.

Analysis of the product of APH(3′)-Ia turnover by ^{1}H-, ^{31}P-, and ^{13}C-NMR and mass spectrometry indicated phosphorylation of kanamycin A at the 3′-hydroxyl group. Comparison of the hydrogen spectrum (D_2O, pH 9.5) of kanamycin A and kanamycin A 3′-phosphate showed the appearance of a multiplet at 4.1 ppm, indicative of the splitting of kanamycin A 3′-phosphate axial 3′H with axial 2′H and 4′H and three-bond coupling with phosphorus as reported by McKay *et al.* (1994). The single resonance observed at 4.52 ppm in the ^{31}P-NMR spectrum corresponds to that observed for a single tetrahedral phosphate monoester (Gorenstein, 1984). The ^{13}C chemical shifts and mass spectral data were also consistent with the 3′-hydroxyl phosphorylation.

DISCUSSION

As described earlier, the catalytic function of APH(3′)-Ia is a major cause of resistance to a host of aminoglycoside antibiotics. In an effort toward understanding the general properties of this protein, we have purified it. The protein is a monomer in the presence of a thiol reagent but dimerizes slowly in its absence. The addition of thiol converts the dimer to a monomer readily; therefore, we conclude that dimerization is due to oxidation of two cysteine thiols. Fragmentation of the protein by trypsin allowed for detection of a 27 kDa fragment, which lacked the N-terminal portion spanning residues Met-1−Arg-42. The 27 kDa fragment failed to dimerize, suggesting that Cys-10—the only cysteine in this portion of the protein—is the residue responsible for dimerization.

A number of features of the turnover chemistry of this enzyme are of interest. As judged by the values for k_{cat}/K_m, the favorable substrates are phosphorylated at 10^7-10^8 M^{-1} s^{-1}, whereas the poorer substrates such as amikacin and butirosin A are phosphorylated at 10^6 M^{-1} s^{-1}. In effect, all of these substrates are phosphorylated efficiently. However, as summarized in Table 2, only amikacin and butirosin A show any antibacterial activity (MIC of 4−8 μg/mL) against the strain harboring APH(3′)-Ia. The k_{cat} values for all these substrates are at most within 4-fold of each other. Furthermore, the k_{cat} values do not change much as a function of pH within the range of 6.0−8.5. Similarly, the K_m for favorable substrates are in the range of 1−10 μM, but for the poorer substrates, amikacin and butirosin A, the values are 83 and 40 μM, respectively. Both amikacin and butirosin A have their N_1 acylated by the *S*-4-amino-2-hydroxybutyryl (AHB) group, which is believed to disfavor interactions with the enzyme active site. In light of the efficient turnover of the poorer substrates by APH(3′)-Ia, we conclude that these molecules may be transported relatively poorly into the cell, where concentration of the drugs would not be sufficiently high to give saturation for APH(3′)-Ia; hence, the full catalytic potential for the enzyme is not realized. We point out that the bacterium is killed with MICs of 13.6 and 5.3 μM (8 and 4 μg/mL) for amikacin and butirosin A, respectively; these values for amikacin and butirosin A are well below their corresponding K_m values for APH(3′)-Ia.

We have isolated the turnover product of APH(3′)-Ia phosphotransferase activity and demonstrated that kanamycin A is specifically phosphorylated at its 3′-hydroxyl group by APH(3′)-Ia. Furthermore, we have shown that APH(3′)-Ia and APH(3′)-IIa both possess a basal "ATPase" activity, at K_m values for Mg^{2+}ATP comparable to those for the transfer of phosphate to aminoglycosides. This indicates that "ATPase" activity proceeds at physiological condition with *in vivo* concentrations of 3.0−3.3 mM ATP (Findly *et al.*, 1994; Bochner & Ames, 1982); however, its rate is as much as 10^4-10^6-fold slower than the phosphotransferase activity with aminoglycosides as the phosphate acceptor.

Finally, we have attempted to gain insight into the mechanism of the phosphotransferase activity. The phosphotransferase reaction of APH(3′)-Ia involves two substrates, the aminoglycoside and the nucleotide, both of which are transformed into two turnover products.

$$\text{kanamycin A} + Mg^{2+}\text{ATP} \xrightarrow{\text{APH(3′)-Ia}} \text{kanamycin A 3′-phosphate} + Mg^{2+}\text{ADP}$$

Two mechanisms typical for two-substrate systems such as APH(3′)-Ia include the ping-pong mechanism and ordered mechanisms. The ordered mechanisms can be divided further into Theorell−Chance, rapid−random, and ordered−sequential mechanisms. These mechanisms can be distinguished from one another by specific kinetic analyses. A common method used for two-substrate kinetic mechanism analysis is initial velocity diagnostics. Our initial velocity measurements with a number of aminoglycoside substrates (*i.e.*, kanamycin A, lividomycin A, neomycin B, and amikacin) with different K_m values did not provide conclusive secondary plots. Two alternative methods used to determine two-substrate kinetic mechanisms involve product-inhibition and alternative substrate kinetics experiments. The results discussed herein provide evidence that catalysis by APH(3′)-Ia proceeds via an equilibrium-random mechanism.

Both kanamycin A 3′-phosphate and Mg^{2+}ADP were studied as product inhibitors of APH(3′)-Ia. Kanamycin A 3′-phosphate was shown to be a poor inhibitor, and we were unable to use it for successful analysis of mechanism. Product-inhibition experiments for APH(3′)-Ia with Mg^{2+}-ADP showed competitive inhibition against Mg^{2+}ATP. The equilibrium-random mechanism allows interaction of either of the turnover products, kanamycin A 3′-phosphate or Mg^{2+}-ADP, with the enzyme. In most situations, the product will show stronger competitive inhibition against its parent compound, in this case, Mg^{2+}ADP against Mg^{2+}ATP; however, for a true equilibrium-random mechanism, Mg^{2+}-ADP should also exhibit competitive inhibition against kanamycin A. Product inhibition by Mg^{2+}ADP with respect to kanamycin A was not readily measured; hence, accurate analysis was unsuccessful. To provide a more definitive answer to these mechanistic possibilities, the method of alternative substrate kinetic diagnostics was applied (Radika & Northrop, 1984).

For analysis of the kinetic mechanism of a two-substrate enzyme reaction, one routinely uses initial velocity kinetics, which would involve determining the velocities (V) at concentrations of aminoglycoside and nucleotide within their respective K_m ranges. Individual initial velocity determinations for APH(3′)-Ia were attempted with ATP (the presumed nucleotide substrate) as the fixed-concentration substrate and kanamycin A, neomycin B, lividomycin A, or amikacin as the the variable-concentration substrate. The resulting double-reciprocal plots of rate versus aminoglycoside concentration at four fixed concentrations of ATP (50−200 μM)

did not provide conclusive results indicative of any kinetic mechanism, even with amikacin, whose K_m value is over 10-fold higher than those of kanamycin A, neomycin B, and lividomycin A.

The use of alternative substrate diagnostics, where the kinetic patterns are achieved by changing the identity of the fixed substrate rather than its concentration, has been reported to be capable of distinguishing between sequential and ping-pong mechanisms, as well as among the sequential mechanisms themselves (Radika & Northrop, 1984; Gates & Northrop, 1988). The results from the alternative substrate kinetics follow the trend given for the equilibrium-random mechanism. In this type of mechanism, both k_{cat} and K_m values are affected by using alternative substrates (*i.e.*, kanamycin A, neomycin B, and amikacin for alternative aminoglycosides; Mg^{2+}ATP, Mg^{2+}GTP, and Mg^{2+}UTP for alternative nucleotides). Whether the nucleotide (ATP) is the variable-concentration substrate and alternative aminoglycosides (kanamycin A, neomycin B, and amikacin) are the fixed-concentration substrates or, vice versa, where the aminoglycoside (amikacin) is the variable-concentration substrate and alternative nucleotides (Mg^{2+}ATP, Mg^{2+}GTP, and Mg^{2+}UTP) are the fixed-concentration substrates, the lines obtained from the steady-state double-reciprocal plots intersect (*i.e.*, nonparallel, Figures 3 and 4). Hence, this first kinetic evaluation of the mechanism for APHs revealed that the mechanism follows an equilibrium-random mechanism, indicating that the phosphotransferase activity of APH(3′)-Ia is independent of the order of substrate binding to the active site.

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