Spectinomycin Kinase from *Legionella pneumophila*

CHARACTERIZATION OF SUBSTRATE SPECIFICITY AND IDENTIFICATION OF CATALytICALLY IMPORTANT RESIDUES*

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The bacterium *Legionella pneumophila* is the responsible agent for Legionnaires’ disease and has recently been shown to harbor a gene encoding a kinase that confers resistance to the aminoglycoside antibiotic spectinomycin (Suter, T. M., Viswanathan, V. K., and Cianciotto, N. P. (1997) *Antimicrob. Agents Chemother.* 41, 1385–1388). We report the overproduction, purification, and characterization of this spectinomycin kinase from an expressing system in *Escherichia coli*. The purified protein shows stringent substrate specificity for spectinomycin with $K_m = 21.5 \mu M$ and $k_{cat} = 24.2 \text{s}^{-1}$ and does not bind other aminoglycosides including kanamycin, amikacin, neomycin, butirosin, streptomycin, or apramycin. Purification of spectinomycin phosphate followed by characterization by mass spectrometry and $^1$H, $^13$C, and $^{31}$P NMR established the site of phosphorylation of the hydroxyl group at position 9. Thus this enzyme is designated APH(9)-Ia (where APH is aminoglycoside kinase). The enzyme was inactivated by the electrophilic ATP analogue 5'-[p-(fluorosulfonyl)benzoyl]adenosine, consistent with a nucleophilic residue such as Lys lining the nucleotide binding pocket. Site-directed mutagenesis of Lys-52 and Asp-212 to Ala confirmed that these residues were important for catalysis, with Lys-52 playing a potential role in ATP binding and Asp-212 in phosphoryl transfer. Thio and solvent isotope effect experiments in the presence of either Mg$^{2+}$ or Mn$^{2+}$ were consistent with a kinetic mechanism in which phosphate transfer does not contribute significantly to the rate-limiting step. These results establish that APH(9)-Ia is a highly specific antibiotic resistance kinase and provides the requisite mechanistic information for future structural studies.

The rise in immunocompromised individuals over the past several years, coupled with the steady expansion of human activity in previously undeveloped ecological niches, has resulted in an increase in the incidence of infections caused by novel as well as traditionally non-pathogenic organisms. One of the best known of these “emerging infections” is associated with the bacterium *Legionella pneumophila*, the causative agent for Legionnaires’ disease (1). This organism is a ubiquitous aquatic protozoan parasite that becomes air-borne in aerosols, and thus water distribution systems which are conducive to aerosol formation (e.g. showers and air conditioning towers) are potential sources of the infectious agent. Inhalation of the aerosols by susceptible individuals, which include the elderly and those with impaired pulmonary or immune systems, can lead to respiratory disease (pneumonia) and, if untreated in its severest form, can result in death (2).

*L. pneumophila* is a Gram-negative bacillus that is commonly found as a freshwater protozoan parasite (3). Infection in humans occurs primarily by intracutaneous colonization of phagocytes (1). The organism is susceptible to most antibiotics on agar plates but, when colonizing protozoa or phagocytes, is resistant to many of these agents, presumably due to difficulties of the drugs in reaching their targets. In the clinic, infection is generally controlled by treatment with erythromycin, rifampicin, and/or a fluoroquinolone (4).

Despite the problem of drug delivery to the bacterium, the lack of intrinsic resistance to antibiotics has been of great benefit for the development of effective treatment regimens. Recently, however, it was shown that *L. pneumophila* from several serogroups encode a chromosomal spectinomycin (Spec) resistance gene designated *aph* (5). The *aph* gene lacks both an obvious promoter region and ribosome-binding site which led to the speculation that the gene could be part of an operon (5). The gene encodes a 331-amino-acid protein with a predicted mass of 38.5 kDa. Homology search indicated that the protein was related to the aminoglycoside kinases (APH) that are important resistance determinants in both Gram-negative and Gram-positive pathogens (6). Indeed, expression of the *aph* gene in *Escherichia coli* followed by assay for Spec kinase activity confirmed the predicted activity and also indicated that the enzyme was quite specific in that other aminoglycosides including kanamycin, streptomycin, and hygromycin were not substrates, at least for enzyme in crude cell lysates (5).

Bacterial resistance to Spec occurs principally via adenylation of the hydroxyl group at position 9 (Fig. 1). Two enzyme families that confer this resistance are distributed in both Gram-negative and Gram-positive organisms. The enzyme ANT(3’)-IIa is distributed among the Enterobacteriaceae and confers resistance to both Spec and streptomycin (7), whereas ANT(9)-Ia and -Ib are exclusive Spec adenylyltransferases

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1. The abbreviations used are: Spec, spectinomycin; APH, aminoglycoside kinase; ATP-S, adenosine 5’-O-(3’-phosphate); FSBA, 5’-O-(fluorosulfonyl)benzoyladenosine; PCR, polymerase chain reaction; CHES, 2-cyclohexylaminoethanesulfonic acid; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; FID, free induction decay(s); ANT, aminoglycoside adenylyltransferase.
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Preparation of Overproducing Constructs

Construction of an APH expression plasmid was achieved by PCR amplification of the corresponding gene found on plasmid pJLJ4 (5) using Vent DNA polymerase (New England Biolabs, Beverly, MA). The 5'-PCR primer incorporated a unique NdeI site (underlined), 5'-GCTTCTAGACATATGCTCAAACAAACCA-3', and the 3'-PCR primer incorporated a unique HindIII site (underlined) 5'-CGAAGCTTTGGATCC-3'. The amplified gene was cloned with NdeI and HindIII and cloned into similarly cleaved pET-22b(+) (Novagen, Madison, WI) to give plasmid pETLP7. The aph(9)-Ia gene was completely sequenced to ensure that no undesired mutations had resulted during PCR amplification.

Site-directed Mutagenesis of Lys-52 and Asp-212 to Ala

The mutagenic oligonucleotides 5'-GCCCGCATGATAGCAAGATGACATAGACATATGCTCAAACAAACCA-3' and 5'-CGAAGCTTTGGATCC-3' were incorporated into the respective Asp-212 → Ala and the Lys-52 → Ala mutants, by the Quick-Change mutagenesis method (Stratagene, La Jolla, CA). The presence of the desired mutations was confirmed by DNA sequencing, and the entire gene was then sequenced to ensure no additional mutations had occurred. The resulting constructs, pETLPD212A4 and pETLPK52A4, were subsequently cloned into E. coli BL21(DE3) for protein purification.

Protein Purification

Wild type and mutant APH(9)-Ia proteins were similarly purified at 4 °C.

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\text{APH(9)-Ia from E. coli BL21(DE3)/pETLP7—Four 1-liter solutions of Luria broth were each separately inoculated with 10 ml of an overnight culture of E. coli BL21(DE3)/pETLP7 and incubated at 37 °C and 250 rpm in the presence of 100 μg/ml ampicillin until the culture reached an absorbance of 0.6 at 600 nm. A solution of boiling sterile medium (150 ml) was then added to raise the culture temperature to ~42 °C in an effort to induce the expression of chaperonins. The cultures were then incubated at room temperature for 10 min, and isopropl-β-d-thiogalactopyranoside was then added to a final concentration of 0.5 mM. The cells were grown for an additional 3 h at 30 °C (250 rpm) to an absorbance of 1.5 at 600 nm, harvested by centrifugation at 4000 × g, and washed with 0.85% NaCl chilled to 4 °C. The cells were resuspended in 15 ml of lysis buffer (50 mM CHES, pH 9.0, 100 mM NaCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol) and disrupted by three passages through a chilled French pressure cell at 20,000 p.s.i. The lysate was clarified by centrifugation at 20,000 × g, and the pellet fraction was re-extracted with an additional 10 ml of lysis buffer and centrifuged at 20,000 × g, and the supernatants were combined. The lysate was then diluted to 45 ml with buffer A (50 mM CHES, pH 9.0, 1 mM EDTA) and applied to a Q-Sepharose (Amersham Pharmacia Biotech, Montreal, Quebec, Canada) column (2.6 × 11.5 cm). APH(9)-Ia was eluted by a fast linear gradient of buffer B (50 mM CHES, pH 9.0, 1 mM EDTA, 1 mM NaCl) from 0–30% over 2.5-column volumes. Fractions were analyzed by enzyme assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Spec kinase activity and purity of APH(9)-Ia. Peak fractions were pooled, concentrated, dialyzed overnight against buffer A, and applied to a smaller Q-Sepharose column (1 × 10 cm). APH(9)-Ia was eluted with a slow 0–30% gradient in buffer B over 5 column volumes. Peak fractions were similarly analyzed, and the purest fractions were pooled, dialyzed against 25 mM KH₂PO₄, pH 7.5, and applied to a Macro-Prep Ceramic Hydroxyapatite type I column (10 × 64 mm) (Bio-Rad). APH(9)-Ia was eluted with a linear gradient from 25 to 1000 mM KH₂PO₄, pH 7.5. Pure fractions of APH(9)-Ia were pooled and stored at 4 °C in 50 mM Tris-HCl, pH 8.0.

Mutant APH(9)-Ia Proteins—APH(9)-Ia D212A and APH(9)-Ia K52A were purified as described above for wild type APH(9)-Ia with the exception of the hydroxyapatite step in the purification of APH(9)-Ia K52A. In this case, the hydroxyapatite step was omitted, and the pooled fractions from step two were applied to a Resource Q (1 ml) (Amersham Pharmacia Biotech, Montreal, Quebec, Canada) and eluted with a linear gradient from 0 to 30% buffer B.

Enzyme Assay

Enzyme activity was monitored by coupling the release of ADP from the Spec kinase reaction to pyruvate kinase/lactate dehydrogenase as described previously for APH(3′)′-IIa (19). Initial rates were fit by nonlinear least squares methods to Equation 1 or to Equation 2 where

Materials and Methods

Reagents

Spec, streptomycin, kanamycin A, neomycin B, butirosin, amikacin, ATP, and 5'-fluorosulfonylbenzoyladenosine (FSBA) were obtained from Sigma. Apramycin was the generous gift of Dr. Astrid Petrich (St. Joseph's Hospital, Hamilton, Ontario, Canada).

Cloning of the aph gene from L. pneumophila and construction of plasmid pJLJ4 have been described previously (5). Restriction enzymes were from MBI Fermentas (Flamborough, Ontario, Canada).

2 D. M. Daigle, G. A. McKay, and G. D. Wright, submitted for publication.
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substrate inhibition was a factor, using the Grafit version 3.0 software (20).

\[ v = \left( k_{\text{cat}}/E_0[S]/(K_m + [S]) \right) \]  \hspace{1cm} (Eq. 1)

\[ v = \left( k_{\text{cat}}/E_0[S]/(K_m + [S][1 + [S]/K_i]) \right) \]  \hspace{1cm} (Eq. 2)

Various aminoglycoside antibiotics were tested for their ability to act as either substrates or inhibitors of APH(9)-Ia. The affinity of APH(9)-Ia to phosphorylate potential aminoglycoside substrates was assessed using the enzyme assay described above and the aminoglycoside and ATP being fixed at 1 mM and 930 \( \mu M \), respectively. The ability of these aminoglycosides to act as inhibitors of Spec kinase activity was also determined using the coupled assay with both Spec and the potential aminoglycoside substrates fixed at 100 \( \mu M \) and ATP fixed at 900 \( \mu M \). Spectinomycin or 160 scans for spectinomycin phosphate. The fixed delays during the pulse sequence were a 1.0-s relaxation delay and a polarization transfer delay of 0.001786 s. Spec inactivation experiments were carried out in 50 mM Tris-HCl, 10 mM MgCl\(_2\), and 0.1 mM FSBA dissolved in dimethyl sulfoxide (1 \% v/v final concentration). Inactivation reactions were allowed to proceed for various lengths of time at 37 \( ^\circ \)C, and any unreacted FSBA was removed by passage through a Sephadex G-10 spin column (0.4 ml) and aliquots assayed for enzymatic activity at room temperature. The rate of inactivation was determined by fitting to a first order rate equation using the Grafit version 3.0 software (20).

Preparation and Purification of Phosphorylated Spec

Phosphorylated Spec was synthesized in vitro by the incubation of 200 \( \mu M \) of Spec and a 3-fold molar excess of ATP (896 \( \mu M \)) in 50 mM Tris-HCl, pH 7.5, and 10 mM MgCl\(_2\). The reaction allowed to proceed for a further 8 h. The reaction mixture was added batchwise to a slurry of AG-50W-X8 (Bio-Rad) (NH\(_4\)) form) and allowed to incubate with stirring for 30 min. The liquid was decanted; the slurry washed with 500 ml of H\(_2\)O, and the liquid fractions were combined and lyophilized. The residue was dissolved in 40 ml of H\(_2\)O and aciddified with 3 drops of 6 \( \% \) HCl. The sample was then added to a AG-50W-X8 column (NH\(_4\)) form) (1 \times 10 cm) which had been pre-equilibrated with 0.5 mM HCl. Phosphorylated Spec-phosphate was eluted with a 0–300 mM linear gradient of NH\(_4\)OH. Fractions were collected and analyzed by thin layer chromatography (19), and those that were judged to contain phosphorylated Spec were pooled and lyophilized. The white solid was taken up in 3 ml of H\(_2\)O, and 500-\( \mu \)l aliquots were applied separately to an H\(_2\)O-equilibrated Mono Q (HR 5/5) column (Amersham Pharmacia Biotech, Montreal). Phosphorylated Spec did not bind to the column, and the pure compound was recovered by lyophilization.

NMR and Mass Spectral Analysis of Phosphorylated Spec

All NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. The compounds used in this study were dissolved in 99.996 \( \% \) D\(_2\)O (Isotec, Inc.) to a concentration of approximately 25 mg/ml for spectinomycin and 5 mg/ml for spectinomycin phosphate. Chemical shifts are reported in ppm relative to tetramethylsilane using the residual solvent signals at HDO at 4.66 ppm as internal reference for the \(^1\)H. Carbon-13 spectra were referenced relative to external dioxane residual solvent signals at HDO at 4.60 ppm as internal reference for shifts are reported in ppm relative to tetramethylsilane using the spectral width. The \(^1\)H 90 \( ^\circ \) pulse width was 6.6 \( \mu s \) (35 \( ^\circ \) flip angle) and a relaxation delay of 0.5 s. The 1H and 13C NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer. The compounds used in this study were dissolved in 99.996 \( \% \) D\(_2\)O at 0.0 ppm.

Proton spectra were acquired at 500.130 MHz using a 5-mm broadband inverse probe with triple axis gradient capability. Spectra were obtained in 32 scans over a 3.858-KHz spectral width (4.247-s acquisition time). Sample temperature was maintained at 30 \( ^\circ \)C by a Bruker Eurotherm variable temperature unit. The free induction decays (FID) were processed using Gaussian multiplication (line broadening, -1.5 Hz; Gaussian broadening, 0.2) and were zero-filled to 64K before Fourier transformation.

Proton COSY two-dimensional NMR spectra were recorded in the absolute value mode using the pulse sequence 90\(^\circ\)-t1-45\(^\circ\)-acquire and included pulsed field gradients for coherence selection. Spectra were acquired in 1 scan for spectinomycin or 8 scans for spectinomycin phosphate for each of the 256 FIDs that contained 2K data points in F2 over the previously mentioned spectral width. The \(^1\)H 90 \( ^\circ \) pulse width was 6.6 \( \mu s \) (35 \( ^\circ \) flip angle) and a relaxation delay of 1.0 s. A 1.0-s relaxation delay was employed between acquisitions. Zero-filling in F1 produced a 1K \times 1K data matrix with a digital resolution of 3.768 Hz/point in both dimensions. During two-dimensional Fourier transformation a sine-bell squared window function was applied to both dimensions. The transformed data were then symmetrized.

Carbon-13 NMR spectra were recorded at 125.758 MHz using the 5-mm broadband inverse probe with triple axis gradient capability. The spectra were acquired over a 28.855-KHz spectral width in 32K data points (0.557-s acquisition time). Single pulse spectra were obtained with a 13C pulse width of 5.0 \( \mu s \) (35 \( ^\circ \) flip angle) and a relaxation delay of 0.5 s. The FIDs were processed using exponential multipletation (line broadening, 4.0 Hz) and zero-filled to 64K before Fourier transformation. Inverse detected \(^1\)H-\(^{13}\)C two-dimensional chemical shift correlation spectra were acquired in the phase-sensitive mode using the pulsed field gradient version of the HSQC pulse sequence. The FIDs in the F2 (\(^1\)H) dimension were recorded over a 2.858-KHz spectral width in 1K data points. The 128 FIDs in the F1 (13C) dimension were obtained over a 13.333-KHz spectral width. Each FID was acquired in 4 scans for spectinomycin or 160 scans for spectinomycin phosphate. The 90 \(^\circ\) 1H pulse was 6.6 \( \mu s \), while the 13C 90 \(^\circ\) pulse was 11.6 \( \mu s \). The data were processed using a sine-bell squared window function shifted by \( \pi/2 \) in both dimensions and linear prediction in F1 to 256 data points followed by zero-filling to 1K.

The pulsed field gradient version of the HMBC pulse sequence was used to acquire inverse detected \(^1\)H-\(^{13}\)C two-dimensional chemical shift correlation spectra through two- and three-bond coupling interactions in the absolute value mode. The FIDs in the F2 (\(^1\)H) dimension were recorded over a 2.858-KHz spectral width in 1K data points. The 128 FIDs in the F1 (13C) dimension were obtained over a 13.333-KHz spectral width. Each FID was acquired in 16 scans for spectinomycin or 224 scans for spectinomycin phosphate. The fixed delays during the pulse sequence were a 1.0-s relaxation delay and a polarization transfer delay of 0.001786 s. The 90 \(^\circ\) 1H pulse was 6.6 \( \mu s \), while the 13C 90 \(^\circ\) pulse was also 11.6 \( \mu s \). The data were processed using a sine-bell squared window function shifted by \( \pi/2 \) in both dimensions and linear prediction to 256 data points in F1 followed by zero-filling to 1K.

Phosphorus-31 NMR spectra were recorded at 292.456 MHz using the 5-mm broadband inverse probe with triple axis gradient capability. The spectra were acquired over a 48.5445-KHz spectral width in 32K data points (0.338 s acquisition time). Single pulse spectra were obtained with a 13P pulse width of 3.0 \( \mu s \) (35 \( ^\circ \) flip angle) and a relaxation delay of 0.5 s. The FIDs were processed using exponential multipletation (line broadening, 2.0 Hz) and zero-filled to 64K before Fourier transformation. Electrospray mass spectra were obtained in the presence of either trifluoroacetic acid or NH\(_4\)OH with a Fison platform quadrupole spectrometer at the McMaster Regional Center for Mass Spectrometry.

Miscellaneous Methods

DNA sequencing was performed at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Protein concentrations were determined by the method of Bradford (22).

RESULTS

Overexpression and Purification of APH(9)-Ia—The Spec kinase from L. pneumophila expressed well in E. coli and resulted in the expected Spec\(^{\beta}\) phenotype. The construct described here, pETL7P, gave approximately 5 mg of pure enzyme from a 1-liter culture (Table I, Fig. 2). Initial difficulties in the yield of enzyme were overcome by gradually raising the culture temperature to 42 \( ^\circ \)C immediately prior to induction by isopropyl-\(\beta\)-thiogalactopyranoside followed by growth at 30 \( ^\circ \)C. We speculate that the improvement in soluble enzyme quantity may be the result of induction of chaperonins by the heat shock step.

Substrate Specificity of APH(9)-Ia—Purified recombinant
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The 1H spectrum of spectinomycin in D$_2$O was composed of four spin systems arising from the protons on carbons 2 and 3 and those on carbons 5a through 9a (Table III and Table IV). The isolated proton on C-10a appeared as a slightly broadened singlet at 4.740 ppm. Singlets from the N-CH$_3$ groups were at 3.132 ppm. Finally, both 4.176 and 3.836 ppm where coupled with the doublet of doublets centered at 4.636 ppm, and this signal was therefore readily identified as the doublet at 1.122 ppm. In the COSY spectrum the C-2 methyl displayed a correlation with the complex multiplet centered at 3.862 ppm (H-2). The H-2 multiplet displayed a cross-peak with the group of 3 main signals located between 1.69 and 1.76 ppm. These signals, attributed to the C-3 methylene protons, collapsed into a broadened singlet centered at 1.731 ppm when the H-2 multiplet was irradiated during a spin decoupling experiment. The unusual appearance of the C-3 methylene protons arose due to their being the AB part of a degenerate ABX spin system. These spin systems generally have fewer than the expected 8 lines in the AB part of spectrum (23).

The protons on the ring formed by carbons 5a through 9a were resolved in the 3.0–4.6 ppm region of the spectrum. However, it was initially difficult to obtain definitive chemical shift assignments. An examination of the spectinomycin structure (24) indicated that the proton on C-7 had an equatorial geometry, whereas all the others were axial. The multiplet that contained only small coupling constants ($^3$$J = 2.8$ Hz) was the doublet of doublets at 4.636 ppm, and this signal was therefore assigned to H-7. In the COSY spectrum H-7 was correlated with the multiplets at 3.371 and 3.132 ppm. These protons must arise from those on C-6 and C-8. However, the similarity in the nature of the substituents on these carbons made it difficult to obtain a definite assignment of H-6 and H-8 based on chemical shifts alone. It was uncertain if H-6 was assigned to 3.371 or 3.132 ppm and similarly for H-8. The COSY spectrum showed a cross-peak between the 3.371 ppm signal and the doublet of doublets centered at 4.176 ppm, whereas the 3.132 ppm resonance was correlated with the signal at 3.921 ppm. Since the two connectivity pathways that started at H-7 (4.636 ppm) and proceeded through 3.371 to 4.176 ppm and 3.132 to 3.836 ppm both ended at 3.921 ppm, this indicated that 3.921 ppm be assigned to the ring junction proton H-9a. Additional support for the H-9a assignment was provided by recording a 1H NMR spectrum with a narrower spectral width in order to improve the digital resolution. Under these conditions the C-10a proton was split into a doublet with a 0.6-Hz coupling constant. This splitting also appeared on the transitions of the 3.921 ppm signal which could now be assigned to H-9a. A 4-bond spin coupling between H-10a and H-9a would account for these observations. A closer examination of the COSY spectrum revealed a weak cross-peak between H-10a and H-9a.

The $^{13}$C NMR spectrum was then acquired to determine if the $^{13}$C chemical shifts would allow a definitive assignment of the position 6 and 5a and 8 and 9 carbons and protons (Table III). Carbon chemical shift assignments were obtained from HSQC and HMBC two-dimensional shift correlation experiments. The one-bond correlations observed in the HSQC spectrum provided the assignment of carbons 2, 3, the C-2 methyl group, and C-10a (Table III). Carbon-7 was assigned to 59.7 ppm, whereas C-9a could be assigned to 69.9 ppm. Again the similarity in $^{13}$C chemical shifts of the signals from carbons 5a, 6, 8, and 9 did not permit their definite assignment. The HSQC data did provide the correlation between these carbons and their directly bonded protons.

An HMBC experiment was then performed with the delay time ($1/2J = 0.88$ s) optimized for a 6.25-Hz long range $^1$H–$^{13}$C spin coupling. The data contained in the HMBC spectrum...
permitted the assignment of the quaternary carbons 4 and 4a to 93.6 and 91.9 ppm, respectively. Carbon-4 was differentiated by its correlation with the protons on C-3, C-2, and with those on the C-2 methyl. In contrast the 91.9 ppm quaternary carbon displayed correlations with only the C-10a proton at 9.47 ppm and the C-3 methylene protons. In the other ring, the assignment of C-9a to 69.9 ppm was confirmed by the observation of a 3-bond correlation with the C-10a proton. Carbon-9a also displayed 2-bond correlations with the protons on 4.176 ppm, whereas the carbon at 2.705 ppm.

When the structure and the NMR data of spectinomycin were re-examined, it was found that the key structural element that could provide a definitive assignment of the C-5a, C-6, C-8, and C-9 protons and carbons would be the possible 3-bond range coupling between H-5a and the quaternary carbon C-4a. The HMBC experiment was repeated with the delay time optimized for a smaller long range coupling of 4.5 Hz. In this experiment C-4a did indeed show a clear correlation with the proton at 4.176 ppm which must therefore be assigned to H-5a. The HMBC spectrum was not able to provide sufficient information to again allow a definite assignment of the quaternary carbons 5a and 6 and 8 and 9. However, it did provide the connection between the N-CH groups and a particular ring carbon. The carbon at 58.7 ppm showed a 3-bond correlation with the N-methyl protons at 2.681 ppm, whereas the carbon at 61.7 ppm had a similar correlation with the N-methyl protons at 2.705 ppm.

The 1H–31P coupling, the remaining protons on C-5a, C-6, C-8, and C-9 had a similar correlation with the N-methyl protons at 2.681 ppm, whereas the carbon at 58.7 ppm had a similar correlation with the N-methyl protons at 2.705 ppm.

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regiospecificity has been rigorously determined by NMR methods, and the enzyme is therefore designated APH(9)-Ia. Alignment of the APH(9)-Ia sequence with APH(3\*9)-IIIa reveals that C-terminal region of APH(9)-Ia has at least seven insert regions of greater than 3 amino acids (Fig. 4). This is notable as the C termini of APH(3\*9) enzymes are very conserved, and the crystal structure of APH(3\*9)-IIIa demonstrates that it is highly defined and positioned at the opening of the active site. This placement as well as mutagenesis studies indicate that it plays a significant role in aminoglycoside binding. Thus the larger C-terminal region of APH(9)-Ia probably plays a significant role in determining substrate specificity, and in particular, the terminal 10 or so amino acids that do not align well with APH(3\*9)-IIIa (Fig. 3) may serve as a gateway excluding larger aminoglycosides while still permitting Spec entry.

FSBA inactivates APH(3\*9)-IIIa by covalent modification of either Lys-33 or Lys-44 (21). Amino acid sequence alignment of APH(9)-Ia with other APHs of varied specificity revealed a direct overlap of Lys-52 with Lys-44 of APH(3\*9)-IIIa (Fig. 3). This Lys was covalently modified by FSBA (21) and has been shown to interact with the \(\alpha\) and \(\beta\) phosphates of ADP in the crystal structure of the APH(3\*9)-IIIa-ADP complex (13). These results support a role for Lys-52 in positioning of ATP in the enzyme active site analogous to Lys-44 in APH(3\*9)-IIIa.

**TABLE III**

| Proton/carbon | \(^1\)H ppm | \(^{13}\)C ppm |
|---------------|-------------|-------------|
| 2             | 3.862       | 68.5        |
| 3             | 1.731       | 41.6        |
| 4             | 93.6        |             |
| 4a            | 91.9        |             |
| 5a            | 4.176       | 65.7        |
| 6             | 3.371       | 58.7        |
| 7             | 4.636       | 59.7        |
| 8             | 3.132       | 61.7        |
| 9             | 3.836       | 66.1        |
| 9a            | 3.921       | 69.9        |
| 10a           | 4.740       | 93.6        |
| 2-CH\(_3\)    | 1.122       | 110.8       |
| 6-NCH\(_3\)   | 2.681       | 30.3        |
| 8-NCH\(_3\)   | 2.705       | 31.7        |

* Estimated chemical shift since this is a second order ABX spin system.

**TABLE IV**

| Proton        | \(^1\)H-J (Hz) | \(^1\)H-J (Hz) |
|---------------|---------------|---------------|
| 2,2-CH\(_3\)  | 6.2           | 6.2           |
| 2,3           | 6.3           | 6.0           |
| 5a,5          | 11.0          | 11.0          |
| 5a,9a         | 9.9           | 10.6          |
| 6             | 2.8           | 2.9           |
| 7             | 2.8           | 2.9           |
| 8             | 10.2          | 9.9           |
| 9a            | 10.1          | 9.8           |
| \(^3\)J\(_{\text{H-P}}\) | 0.6         | **TABLE III**

**4^*\)**

**TABLE IV**

| Proton/carbon | \(^{13}\)C ppm |
|---------------|-------------|
| 2             | 68.3        |
| 3             | 68.3        |
| 4             | 93.6        |
| 4a            | 91.6        |
| 5a            | 65.3        |
| 6             | 58.9        |
| 7             | 59.7        |
| 8             | 63.8        |
| 9             | 66.3        |
| 9a            | 69.9        |
| 10a           | 93.6        |
| 2-CH\(_3\)    | 110.8       |
| 6-NCH\(_3\)   | 30.3        |
| 8-NCH\(_3\)   | 31.7        |

* Estimated value of observed coupling constant since part of a second order ABX spin system.

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![Fig. 3. Inactivation of APH(9)-Ia with 0.1 mM FSBA. The relative enzyme activity was determined in the absence (●) and presence (○) of 1 mM ATP.](image)

![Fig. 4. Overlap of APH(9)-Ia with APH(3\*9)-IIIa. The amino acid sequences of 20 APHs were aligned using the program ClustalW (15); only the sequences of APH(9)-Ia and APH(3\*9)-IIIa are indicated for clarity. Regions determined to be of catalytic importance are boxed, and the invariant and similar residues are marked by an asterisk or dash, respectively.](image)
The precise mechanism of phosphoryl transfer is at present unknown for any aminoglycoside kinase. In particular, the role of the conserved active site Asp (Asp-212 of APH(9)-Ia) has not been adequately assessed. That mutation to Ala results in a dramatic loss of enzyme activity is indicative of a critical role in catalysis, but whether it acts as a general base, activating the nucleophilic hydroxyl as indicated in Fig. 5, or plays some other role, is not known. A complicating factor in assessing the mechanism of phosphoryl transfer in the steady state is that product release appears to be largely rate-limiting for these enzymes, thus probing of the group transfer chemistry will require either conditions in which the chemical steps become more rate-limiting or pre-steady state analysis. The latter conditions have recently been used to investigate the mechanism of CAMP-dependent protein kinase (17), but the precise role of the conserved Asp remains elusive.

IMPLICATIONS AND CONCLUSIONS

We have demonstrated that the chromosomal aph gene recently discovered in \textit{L. pneumophila} is a highly specific Spec kinase which phosphorylates this aminoglycoside antibiotic exclusively on the hydroxyl group at position 9. This stringent substrate specificity is atypical for this class of antibiotic resistance enzyme where substrate promiscuity is standard, although it is not unprecedented, for example, the APH(6) enzymes are exclusive streptomycin kinases (6). The site of phosphoryl transfer was determined to be to the axial hydroxyl group at position 9, a position that has been shown to be the location of adenylyltransfer for enzymes with ANT(9) activity. Thus in the face of these precedents, it is likely that the recently cloned Spec kinase from the Spec-producing organism \textit{S. flavopersicus} (10) also shares this specificity, and thus we propose that in the absence of contrary information that the enzyme be classified as APH(9)-Ib.

Finally, some consideration of the biological role of this enzyme is required. The narrow substrate and regio-specificities of the enzyme as well as the catalytic efficiency as assessed by the $k_{\text{cat}}/K_m$ values of 1 $\times$ 10$^{6}$ M$^{-1}$ s$^{-1}$ determined here suggest a highly specific and evolved catalyst. It seems unlikely this \textit{L. pneumophila} enzyme has evolved specifically for the detoxification of Spec, and this reaction may be fortuitous, although the ecology of organism is not well known and it may come into contact with Spec-producing organisms. Perhaps, like the aminoglycoside acetyltransferase AAC(2'-Ia) from \textit{Providencia stuartii} where this enzyme is thought to play a role in peptidoglycan modification (28), APH(9)-Ia may also have an as yet undetermined physiological role phosphorylating other substrates important to \textit{L. pneumophila}.

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