Active-Site Labeling of an Aminoglycoside Antibiotic Phosphotransferase (APH(3′)-IIIa)†

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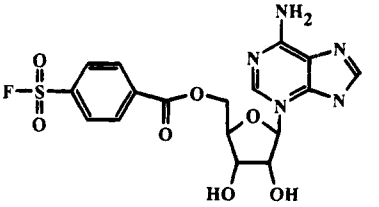
ABSTRACT: The aminoglycoside antibiotics are inactivated by modifying enzymes that are now widely distributed in many pathogenic bacteria. This situation threatens the continued use of these clinically important drugs. We have undertaken studies to understand the molecular mechanism of aminoglycoside resistance, and we report the affinity labeling of the enterococcal aminoglycoside 3′-phosphotransferase, APH(3′)-IIIa, with an electrophilic ATP analogue, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA). Incubation of purified APH(3′)-IIIa with FSBA resulted in time-dependent irreversible inactivation of enzyme activity with a binding constant, K_i, of 0.406 mM and a rate of maximal inactivation, k_{max}, of 0.086 min^{-1}. Addition of ATP completely protected the enzyme from inactivation, consistent with labeling of the ATP binding site. Reaction of APH(3′)-IIIa with [^{14}C]FSBA showed that inactivated APH(3′)-IIIa incorporates 1 mol of FSBA/mol of enzyme. Peptide mapping of FSBA-inactivated APH(3′)-IIIa resulted in the identifiction of two peptide peaks with highly increased absorbance at 260 nm, indicative of covalent labeling with FSBA. Analysis by electrospray ionization mass spectrometry and Edman degradation revealed two tryptic peptides, Val31-Lys44 and Leu34-Arg49, which incorporated the FSBA label at Lys33 and Lys44, respectively. This establishes the importance of the N-terminal region of APHs in ATP binding, a region of these enzymes which has heretofore not been considered for involvement in substrate binding.

The emergence of bacteria resistant to many antibiotics is a medical problem approaching crisis levels (Berkelman & Hughes, 1993; Kunin, 1993). In fact, some are heralding the oncoming of a post-antibiotic era, where drug resistance will again make previously treatable infections life-threatening (Hyde, 1994). Antibiotic resistance in Gram positive cocci is especially troublesome as these organisms are major sources of nosocomial (hospital-acquired) infections (Moellering, 1991; Tomasz, 1994). Treatment of infections caused by enterococci and staphylococci frequently requires administration of aminoglycoside antibiotics. These drugs are targeted to the bacterial 30S ribosomal subunit and interfere with normal translation causing pleiotropic effects that culminate in cell death (Davis, 1987). Bacteria have acquired resistance to high levels of aminoglycosides by the synthesis of proteins that covalently modify the drugs (Davies, 1994).

We have recently overexpressed and characterized the enterococcal aminoglycoside resistance enzyme aminoglycoside 3′-phosphotransferase, APH(3′)-IIIa,[1] which regiospecifically phosphorylates kanamycin in an ATP-dependent fashion (McKay et al., 1994). This enzyme also inactivates the clinically important aminoglycosides amikacin and neomycin. The molecular basis for the wide substrate specificity exhibited by this enzyme is of considerable interest, especially for prediction of the continued effectiveness of aminoglycoside therapy. In addition, knowledge of the molecular mechanism of enzymatic aminoglycoside inactivation, which includes not only the kinetic and chemical mechanisms but also the active-site geometry, can be used to guide the synthesis of inhibitors that could restore the effectiveness of aminoglycosides inactivated by APHs. We have therefore undertaken the identification of regions of the enzyme that are involved in catalysis by several methods including affinity labeling. As a first step we have examined the interaction of an ATP analogue, 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (FSBA) (**1**), with purified APH(3′)-IIIa. This analogue has been shown to label nucleophiles at the active site in many ATP-requiring enzymes [reviewed in Colman (1990)]. We report herein that FSBA covalently labels APH(3′)-IIIa and report the sequence of two labeled peptides which permits localization of part of the ATP binding site to the N-terminal region of the enzyme.



FSBA (1)

MATERIALS AND METHODS

Chemicals. APH(3′)-IIIa was purified from *Escherichia coli* BL21(DE3)/pETSACG1 as previously described

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[1] Abbreviations: APH, aminoglycoside phosphotransferase; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine.

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(McKay et al., 1994). FSBA, kanamycin A, TPCK-treated trypsin, and pyruvate kinase/lactate dehydrogenase mix were from Sigma (St. Louis, MO). Sephadex G-25 was from Pharmacia (Montreal, PQ). [^{14}C]FSBA (radioactive label is incorporated in the purine ring) was from DuPont NEN (Mississauga, ON).

Inactivation of APH(3′)-IIIa with FSBA. Typically, FSBA inactivation experiments were conducted in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ with various amounts of FSBA dissolved in dimethyl sulfoxide (10% v/v final for all FSBA concentrations including controls) in a total volume of 225 μL at 37 °C. Reactions were initiated by the addition of purified APH(3′)-IIIa (4.8 nmol) and aliquots (10 μL) removed and assayed for kanamycin kinase activity. Enzyme activity was measured by a coupled assay (McKay et al., 1994) consisting of 50 mM Tris-HCl, pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM kanamycin A, 2.5 mM phosphoenolpyruvate, 0.7 mM NADH, and 5 μL of pyruvate kinase/lactate dehydrogenase mixture. Assays were conducted at 37 °C while the decrease in absorbance at 340 nm (ϵ_m = 6300 M^{-1} cm^{-1}) was continuously monitored using a Cary 3E UV−vis spectrophotometer.

Determination of the Stoichiometry of FSBA Labeling. APH(3′)-IIIa (60 nmol) was added to a solution of 0.45 mM [^{14}C]FSBA (2.2 μCi/μmol) in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ to give a final volume of 1.5 mL and incubated at 37 °C. Aliquots (100 μL) were removed at various time points and reactions quenched by the addition of 2-mercaptoethanol to 1.35 mM. Unbound label was removed by application to two successive Sephadex G-25 spin columns (400 μL) (Penefsky, 1977). The sample was then assayed for kanamycin kinase activity in duplicate and scintillation counted to determine label incorportion.

Large Scale Affinity Labeling and Peptide Mapping. Large scale inactivations of APH(3′)-IIIa for peptide mapping consisted of enzyme (100 nmol), 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.75 mM FSBA in a final volume of 0.5 mL. A control incubation was carried out without FSBA, and a reaction with [^{14}C]FSBA (1.2 μCi/μmol) was also performed. Reactions were incubated at 37 °C for 3 h, the volumes were then increased to 1.5 mL, and the solutions were dialyzed against 2.25 L of 25 mM NH_4HCO_3, pH 8.3, at 4 °C. The residues were taken up in 100 μL of 8 M urea, 100 mM NH_4HCO_3, pH 8.3, and incubated at room temperature for 15 min followed by the addition of 45.5 μL of 100 mM NH_4HCO_3, pH 8.3. Trypsin (220 μg activated in 1.2 M HCl) was added to each sample, and the digestion was allowed to proceed for 24 h in the dark at ambient temperature. Samples were frozen at −80 °C until further use.

Tryptic digests were analyzed by reverse-phase HPLC using a Spherisorb-ODS2 C18 column (4 mm × 20 cm) at ambient temperature at a flow rate of 0.8 mL/min. Samples were applied in a solution of 0.1% TFA, and peptides were eluted by a gradient in 0.07% TFA in CH_3CN (B). The gradient consisted of 0−35% B over 55 min, 35−75% B over 30 min, and 75−90% B over 15 min. Samples were monitored simultaneously at 214 and 260 nm, and radiolabeled digests were analyzed by scintillation counting of 1 min fractions.

Narrowbore HPLC. Tryptic digests were identically manipulated. Two percent of the digestion solution was diluted to a final volume of 100 μL with 0.06% TFA/H_2O, and 90 μL was injected for separation. Separation of tryptic digests were performed by high-performance liquid chromatography using a Vydac C18 (2.1 mm × 150 mm) reverse-phase column on a Hewlett Packard 1090 HPLC with a 1040 diode array detector. Peptides were eluted at a flow rate of 150 μL/min of 0.06% TFA/H_2O and a gradient of 0% B at 0 min, 5% B at 10 min, 33% B at 63 min, 60% B at 95 min, and 80% B at 105 min, where B is 0.057% TFA/acetonitrile. Fractions from the FSBA-inactivated APH(3′)-IIIa chromatogram were chosen on the basis of absorbance evident at 260 nm. Selected fractions were submitted to Edman microsequencing and mass spectrometry.

Table 1: Inactivation of APH(3′)-IIIa by FSBA

FSBA (0.5 mM)	ATP (mM)	tobramycin (0.21 mM)	phosphotransferase activity (%)[a]
−	0	−	97 ± 0.4
+	0	−	18 ± 2.6
+	0.05	−	53 ± 1.6
+	0.10	−	72 ± 2.7
+	0.5	−	106 ± 3
+	1.0	−	105 ± 5
+	0	+	17.8 ± 0.3
+	1.0	+	106 ± 7

[a] Activity remaining after 60 min of incubation at 37 °C; values are the average of two experiments. Activity was normalized to t = 0 min in the absence of any additions except DMSO. Samples consisted of purified APH(3′)-IIIa (70 μg) in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$ in a final volume of 0.15 mL. Phosphotransferase activity was monitored by the coupled enzyme assay described in Materials and Methods.

Edman Degradation. Automated Edman degradation was performed on an Applied Biosystems 477A protein sequencer using a microcartridge, and cycles were optimized for 30 min cycles. Strategies for the selection of peptide fractions and microsequencing have been previously described (Lane et al., 1991).

Mass Spectroscopy. Mass determination of the isolated peptides was obtained using a Finnigan MAT TSQ-700 triple sector quadrupole mass spectrometer equipped with a Finnigan atmospheric pressure ionization electrospray source. Aliquots from the HPLC fractions were directly infused at a flow rate of 3 μL/min using a sheath fluid of 2-methoxyethanol, a flow of nitrogen sheath gas directed through the outer needle assembly, an electrospray voltage differential of 3−4 kV, and a heated capillary temperature of 220 °C. Mass spectra were acquired by scanning the mass range from m/z 400 to 2000 repetitively every 3 s with 8−16 scans summed for molecular mass deconvolution.

RESULTS

FSBA Labels APH(3′)-IIIa at the ATP Binding Site. Incubation of purified APH(3′)-IIIa with FSBA resulted in inactivation of the enzyme (Table 1). Phosphotransferase activity could not be restored by extensive dialysis or gel filtration, showing that the inactivation was irreversible. Addition of increasing amounts of ATP successfully blocked FSBA-mediated inactivation of APH(3′)-IIIa (Table 1). Incubation with tobramycin, a kanamycin analogue which is a potent competitive inhibitor of kanamycin A (K_i = 2 μM) (McKay et al., 1994), had no effect. These results are consistent with the hypothesis that inactivation by the ATP analogue FSBA was occurring by reaction at the ATP binding site. Phenylmethanesulfonyl fluoride (1 mM), a

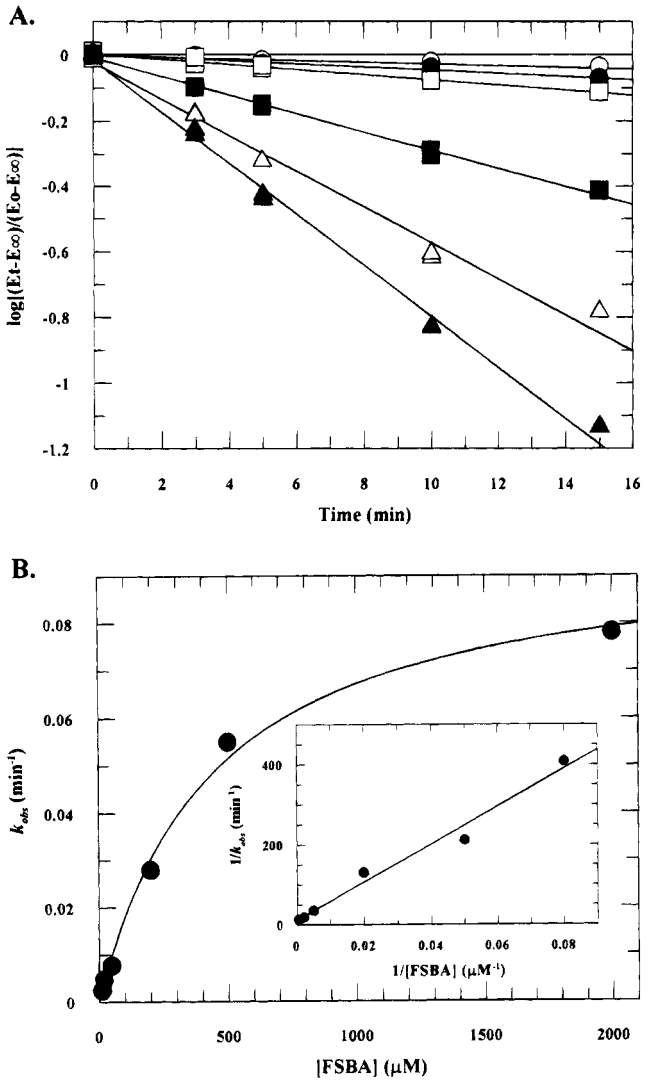


FIGURE 1: Inactivation of APH(3′)-IIIa with FSBA. (A) APH(3′)-IIIa (4.8 nmol) in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, and DMSO (10% final v/v) inactivated with FSBA at a final concentration of 2000 (▲), 500 (△), 200 (■), 50 (□), 20 (●), and 12.5 (○) μM. (B) Plot of first-order rates of inactivation (k_{obs}) vs inactivator concentration. Inset: replot to determine K_i and k_{max}. The correlation coefficient (r) is 0.99.

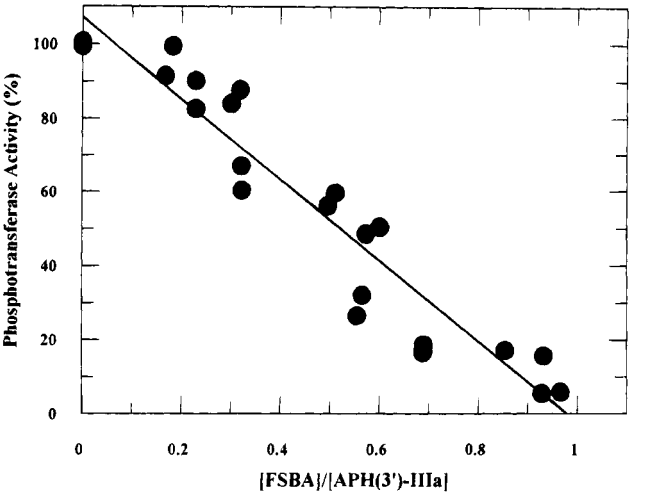


FIGURE 2: Stoichiometry of APH(3′)-IIIa labeling by FSBA. APH(3′)-IIIa was incubated with [^{14}C]FSBA (2.2 μCi/μmol) as described in Materials and Methods. Aliquots were removed at various times and assayed for label incorporation by scintillation counting and enzyme activity by coupled assay. The correlation coefficient (r) is 0.95.

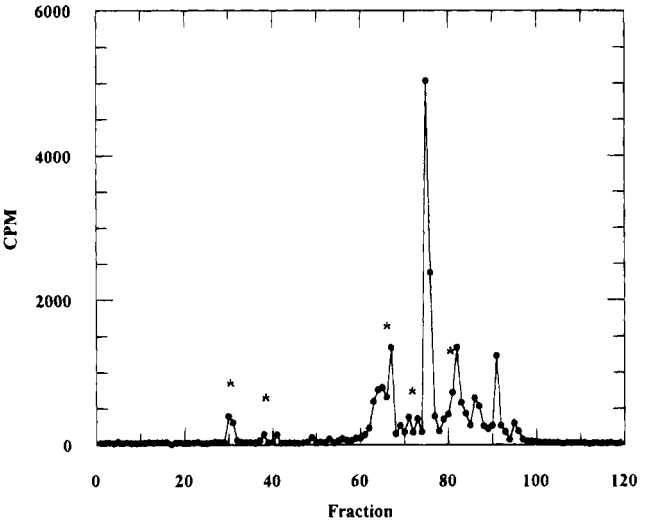


FIGURE 3: Peptide mapping of [^{14}C]FSBA-labeled APH(3′)-IIIa. Peptide separation was performed on a Spherisorb-ODS2 C18 column (4 mm × 20 cm) as indicated in Materials and Methods. Asterisks (*) denote peaks derived from [^{14}C]FSBA only. 0.8 milliliter fractions were collected.

compound with the same reactive group as FSBA, did not inactivate APH(3′)-IIIa under the same conditions. Therefore, nonspecific labeling of reactive APH(3′)-IIIa nucleophiles by FSBA resulting in enzyme inactivation is unlikely.

Further analysis revealed that inactivation was time dependent and consistent with the following reaction:

$$E + I \overset{K_i}{\rightleftharpoons} EI \xrightarrow{k_{max}} EI^* \qquad (1)$$

where K_i is the apparent binding constant, k_{max} is the rate of inactivation at saturating inactivator (I), and EI* is the inactive enzyme. Inactivation of the enzyme was first order with respect to FSBA concentration for the first 10−15 min (Figure 1A) but then appeared to reach a limiting value. Such deviation from nonlinearity with inactivators has been noted by others with similar affinity labels (Tomich & Colman, 1985; Ferrer et al., 1987; Barycki & Coleman, 1993). Addition of fresh FSBA to reactions resulted in further inactivation of APH(3′)-IIIa, indicating that the limiting value was reached because of loss of FSBA by hydrolysis or interaction with buffer, etc. After several additions of FSBA, a final value of 2.5% of the initial activity was consistently reached; we therefore incorporated this value for the remaining enzyme (E_∞) into our calculation of k_{obs}. Since APH(3′)-IIIa is isolated as a mixture of monomer and dimer (McKay et al., 1994), we were concerned that each species could show differing rates of inactivation, though these are catalytically equivalent with respect to phosphorylation of kanamycin (McKay et al., 1994). We saw no difference when the experiments were repeated using purified monomer or dimer.

Kinetic constants may be determined by a replot of the reciprocal of the initial first-order rates (k_{obs}) vs the reciprocal inactivator concentration from eq 2:

$$1/k_{obs} = 1/k_{max} + (K_i/k_{max})(1/[I]) \qquad (2)$$

This permits estimation of the initial binding constant, K_i, to be 406 ± 28 μM, and k_{max}, the maximal rate of inactivation

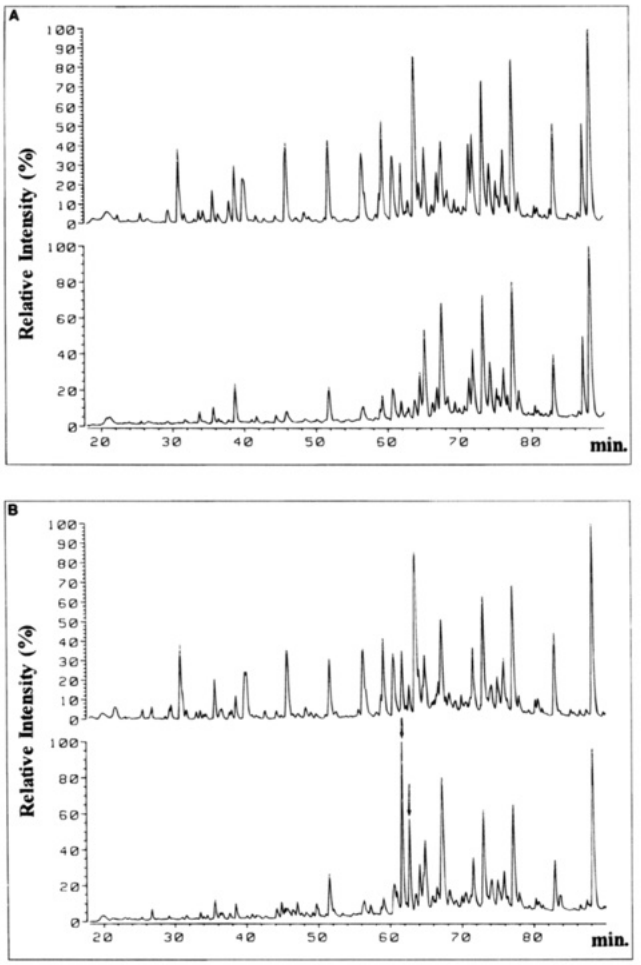


FIGURE 4: Peptide mapping of APH(3′)-IIIa (A) and non-radiolabeled FSBA-inactivated APH(3′)-IIIa (B) by narrowbore reverse-phase HPLC. Separation conditions are detailed in Materials and Methods. Upper trace is the absorbance at 210 nm, and the lower trace is the absorption at 260 nm. Arrows indicate FSBA-labeled peptides which were further analyzed.

at saturating FSBA, to be 0.086 ± 0.077 s^{-1} (Figure 1B). The binding constant is some 13.5 times higher than the K_m for ATP (0.03 mM), suggesting a significant contribution to the binding energy of ATP from the interaction with negatively charged phosphate groups, which are lacking in FSBA, as ATP and FSBA are essentially the same size (Colman, 1990).

Inactivation studies were performed with ^{14}C-labeled FSBA in order to determine the stoichiometry of inactivation. Extrapolation to 100% inactivation occurs when the ratio of APH to covalently labeled FSBA is 0.98 ± 0.03 (Figure 2), supporting our suggestion of one active site per monomer (McKay et al., 1994).

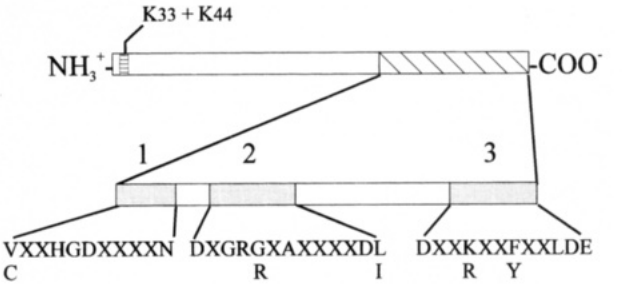
Identification of FSBA-Labeled Peptides. In an effort to precisely locate the site of covalent attachment of FSBA to APH(3′)-IIIa, we performed large scale inactivation (100 nmol) with both ^{14}C-labeled and non-radioactive FSBA followed by tryptic digestion and reverse-phase HPLC separation. Initial experiments using a 4.6 mm internal diameter C18 column showed a strong peak absorbing at 260 nm at ≈77 min for the FSBA-labeled protein but not for the control unlabeled digest. This peak also contained greater than 58% of the total radioactivity (Figure 3), by far the largest radioactive peak observed and the only peak associated with significant new 260 nm absorbance. These

Table 2: Analysis of Tryptic Peptides from FSBA-Labeled APH(3′)-IIIa

retention time[a] (min)	peptide sequence	mass (Da) predicted	mass (Da) ESI-MS	mass difference (Da)
61.7	LFHSIDISDCPYTNSLDSR	2259.4[b]	2258.9	0.5
	VY*LVGENENLYLK	1681.1	2114.8	433.7
62.7	IIELYAECIR	1297.8[b]	1297.9	−0.1
	LVGENENLYL*MTDSR	1881.3	2314.7	433.4

[a] Retention times were obtained following injection of the tryptic digest onto a narrowbore column and identified by their significant absorbance at 260 nm as compared to a control digest done without preincubation with FSBA. Peptides were analyzed by electrospray ionization mass spectrometry and sequenced by Edman degradation as indicated in Materials and Methods. Predicted masses were determined assuming a Lys residue at positions marked with an asterisk as suggested by the primary sequence (Trieu-Cuot & Courvalin, 1983). An asterisk denotes an unidentified amino acid; approximately 10% of predicted Lys was observed. [b] Mass includes an additional disulfide with 2-mercaptoethanol.

Chart 1: Regions of Sequence Homology in APHs[a]



[a] Motifs 1−3 of the C-terminal homology region are indicated. The position of labeled Lys33 and Lys44 of APH(3′)-IIIa are indicated.

experiments demonstrated that the absorbance of FSBA−peptide adducts at 260 nm could be used as a diagnostic for purification of labeled peptides.

Chromatography of tryptic peptides obtained from APH(3′)-IIIa inactivated with non-radioactively labeled FSBA on a reverse-phase narrowbore column (2.1 × 150 mm) revealed only two 260 nm absorbing peaks not found in the unlabeled enzyme (Figure 4). ESI-MS of the peak at 61.7 min revealed two peptides with molecular weights of 2258.9 and 2114.8 (Table 2). N-terminal sequencing indicated two peptides in a ratio of ~3:2. The secondary peptide gave the sequence VY*LVGENENLYLK, where the asterisk indicates a cycle where a PTH-amino acid could not be identified. This corresponds to the tryptic peptide Val31-Lys44 and identifies the unknown amino acid as Lys33. The mass of this peptide as determined by the ESI mass spectrum was 2114.8 Da (Table 2); this value is 433.7 Da higher than predicted for this peptide, consistent with labeling of Lys33 with FSBA (predicted mass of 433.4 Da with loss of F^-). The primary tryptic peptide was identified as Leu121-Arg139. The ESI-MS permitted identification of Cys130 as the 2-mercaptoethanol disulfide.

The second 260 nm absorbing peak at 62.7 min also gave two peptides in a ratio of ~2:1 by Edman microsequencing and two peptides by mass spectral analysis with molecular weights of 1297.9 and 2314.7 (Table 2). N-terminal sequencing of the latter peptide gave LVGENENLYL* MTDSR. The unidentified amino acid corresponds to Lys44 in the primary sequence. The mass of the peptide is 433.4

Chart 2: Alignment of the N-Terminal Regions of APHs[a]

		Reference
APH(3′)-IIIa	G25MSPAKVYKL..VG.ENENLYLK	(Trieu-Cuot& Courvalin,1983)
APH(3′)-Ia	G34QSGATIYRLYGKP.DAPELFLK	(Oka et al., 1981)
APH(3′)-Ib	G34QSGCAVYRLHSKS.GGSDLFLK	(Pansegrau et al.,1987)
APH(3′)-Ic	G34QSGATIYRLYGKP.NAPELFLK	(Lee,et al., 1991)
APH(3′)-IIa	G30CSDAAVFRL.SAQ.GRPVLFVK	(Herbert et al.,1986)
APH(3′)-IVa	G27YSGDHVYHV..KEYRGTPAFLK	(Beck et al., 1982)
APH(3′)-Va	G21DSGAFVYQLTGGPEPQPELYAK	(Thompson & Gray,1983)
APH(3′)-Vb	G20DSGAFVYRLTG...HGPELYAK	(Hoshiko et al.,1988)
APH(3′)-Vc	G20DSGASVYRLAG...QQPELYVK	(Salauze & Davies, 1991)
APH(3′)-VIa	G22QSPSDVYSF..NR.NNETFFLK	(Martin et al.,1988)
APH(3′)-VIIa	G16MSPAEVYKC..QL.KNTVCYLK	(Tenover et al.,1989)

[a] Bullets show invariant residues; asterisks denote similar amino acids. Arrows show the sites of FSBA labeling of APH(3′)-IIIa.

Da larger than predicted, demonstrating labeling by FSBA. The primary peptide consisted of all the expected amino acids in the tryptic peptide Ile111-Arg120, with a predicted mass of 1297.8 Da including 2-mercaptoethanol disulfide with Cys118. Peaks at the same retention time in an unlabeled control tryptic digest were also isolated and, as expected, revealed only the two nonlabeled peptides by Edman microsequencing and ESI-MS analysis (not shown). This combination of mass spectral and N-terminal sequence data unambiguously identifies Lys33 and Lys44 as sites of FSBA reaction and therefore demonstrates their proximity to the ATP binding site.

DISCUSSION

Aminoglycoside antibiotics are inactivated by covalent modification by bacterial enzymes. These include *O*-phosphotransferases, *O*-adenyltransferases, and *N*-acetyltransferases (Umezawa & Kondo, 1982; Shaw et al., 1993). The dissemination of genes encoding these enzymes in a variety of pathogenic bacteria, especially in hospitals as well as day care and other extended care facilities, has impeded the ability to treat infections, particularly in view of the emerging problem of multidrug resistance (Davies, 1994). One of the more prevalent groups of aminoglycoside-inactivating enzymes is the 3′-phosphotransferases, of which seven classes have been identified on the basis of drug resistance phenotype and at least 11 isozymes have been cloned and sequenced (Shaw et al., 1993). Of these enzymes, APH(3′)-IIIa, found in enterococci and staphylococci, shows the widest substrate specificity as determined by the ability of treated bacteria to grow in the presence of aminoglycoside antibiotics and by direct enzyme assay (Trieu-Cuot & Courvalin, 1983; Shaw et al., 1993; McKay et al., 1994).

The APH(3′)s show considerable primary sequence homology which is especially striking in the C-terminal region where three conserved motifs have been noted (Martin et al., 1988; Kirby, 1990; Shaw et al., 1993) (Chart 1). In contrast, the N-terminal region, while showing some conserved residues, is more sequence diverse. This has led to the suggestion that motif 2 of the C-terminal region (corresponding to Gly205-Asp216 in APH(3′)-IIIa) may contain a Gly-rich P-loop that has been noted in many ATP binding proteins (Martin et al., 1988). Indeed, site-directed mutagenesis of conserved residues in motif 2 of APH(3′)-I has been performed, and Arg211 (corresponding to Arg211 of APH(3′)-IIIa) has been implicated in ATP binding on the basis of K_m determinations using crude enzyme samples (Kocabivik & Perlin, 1994). In an effort to gain substantive evidence for localization of the ATP binding region in APH-(3′)-IIIa, we have examined the interaction of purified, overexpressed enzyme with the electrophilic affinity label FSBA.

FSBA is an ATP analogue first described by Colman's group in 1975 (Pal et al., 1975) and has since been used to successfully probe the nucleotide binding sites of a variety of proteins (Zoller & Taylor, 1979; Annamalai & Colman, 1981; Hathaway et al., 1981; Tomich & Colman, 1985; Ferrer et al., 1987; Pettigrew, 1987; Kim et al., 1991). Sulfonyl halides are subject to attack by any nearby nucleophile including the side chains of Lys, Tyr, Cys, His, and Ser [Colman (1990) and references therein]. Given that the position of the reactive group is similar to that of the γ-phosphate of ATP, Lys residues are often labeled as their positively charged side chains are frequently found lining the triphosphate binding site. We find that FSBA inactivates kanamycin phosphotransferase activity and covalently labels APH(3′)-IIIa with a 1:1 stoichiometry. Incubation with ATP but not an aminoglycoside abolishes inactivation, suggesting that FSBA has undergone nucleophilic attack by residue(s) lining the ATP binding site.

Using a strategic combination of peptide mapping by narrowbore HPLC, followed by mass spectral analysis and N-terminal sequencing of labeled peptides, we have identified covalent FSBA-labeling sites on two tryptic peptides which are adjacent on the primary sequence. In both peptides, lysine residues are labeled by FSBA. These residues, Lys33 and Lys44, are located in the extreme N-terminus of the protein and not in the conserved C-terminal motifs. Inspection of this N-terminal region in a number of APH(3′)s reveals a segment with invariant Gly and Ser residues as well as one of the FSBA-labeled lysines, Lys44 (Chart 2).

This strongly suggests that Lys44 is located in the triphosphate region of the ATP binding site of all APH(3′)s and that Lys33 is adjacent in the APH(3′)-IIIa tertiary structure.

The identification of Lys33 and Lys44 as sites of FSBA reaction clearly demonstrates the importance of the N-terminal region in ATP binding of APH(3′)-IIIa and, by extension, all APH(3′) enzymes. This identifies a previously overlooked region of these aminoglycoside-modifying enzymes and suggests new sequences to be probed by other means such as mutagenesis studies to further elaborate the role of this region in catalysis and thus contribute to further structure−function studies and inhibitor design.

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