Aminoglycoside antibiotic phosphotransferases are also serine protein kinases

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Background: Bacterial resistance to aminoglycoside antibiotics occurs primarily through the expression of modifying enzymes that covalently alter the drugs by *O*-phosphorylation, *O*-adenylation or *N*-acetylation. Aminoglycoside phosphotransferases (APHs) catalyze the ATP-dependent phosphorylation of these antibiotics. Two particular enzymes in this class, APH(3′)-IIIa and AAC(6′)-APH(2″), are produced in gram-positive cocci and have been shown to phosphorylate aminoglycosides on their 3′ and 2″ hydroxyl groups, respectively. The three-dimensional structure of APH (3′)-IIIa is strikingly similar to those of eukaryotic protein kinases (EPKs), and the observation, reported previously, that APH(3′)-IIIa and AAC(6′)-APH(2″) are effectively inhibited by EPK inhibitors suggested the possibility that these aminoglycoside kinases might phosphorylate EPK substrates.

Results: Our data demonstrate unequivocally that APHs can phosphorylate several EPK substrates and that this phosphorylation occurs exclusively on serine residues. Phosphorylation of Ser/Thr protein kinase substrates by APHs was considerably slower than phosphorylation of aminoglycosides under identical assay conditions, which is consistent with the primary biological roles of the enzymes.

Conclusions: These results demonstrate a functional relationship between aminoglycoside and protein kinases, expanding on our previous observations of similarities in protein structure, enzyme mechanism and sensitivity to inhibitors, and suggest an evolutionary link between APHs and EPKs.

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Introduction

The second half of the century has seen the discovery, development and exploitation of antimicrobial agents used to treat a wide variety of microorganisms. The use of antibiotics in medicine and agriculture has been paralleled by the selection of bacterial resistance to most of these drugs, a fact that has emerged as a significant crisis in health care [1]. One prevalent form of resistance to antibiotics is enzyme-mediated modification of the drugs. For example, aminoglycoside antibiotics are detoxified by resistant organisms through chemical modification catalyzed by distinct transferases. The family of aminoglycoside antibiotics, which includes kanamycin, gentamicin and many others, can be modified by *O*-phosphorylation, *O*-adenylation and *N*-acetylation [2]. The phosphorylation mechanism involves enzymatic transfer of the γ-phosphate of ATP to an aminoglycoside hydroxyl group. The phosphorylated aminoglycoside no longer functions as an effective chemotherapeutic agent and the organism is therefore able to survive in the presence of aminoglycosides.

One member of this enzyme family, aminoglycoside phosphotransferase type IIIa (APH(3′)-IIIa), has been cloned from *Staphylococcus aureus* [3] and *Enterococcus faecalis* [4], overexpressed in *Escherichia coli* and purified to homogeneity [5]. This enzyme has been shown to inactivate aminoglycosides by phosphorylating the 3′ and/or 5″ hydroxyl group of these antibiotics in an ATP-dependent manner [5,6]. A second aminoglycoside kinase also found in gram-positive cocci, AAC(6′)-APH(2″), is a unique bifunctional enzyme with two functional domains possessing distinct activities. The amino terminus of the enzyme has aminoglycoside acetyltransferase activity, whereas the aminoglycoside kinase activity resides in the carboxy-terminal domain [7]. This enzyme inactivates aminoglycoside antibiotics such as kanamycin by phosphorylation of the 2″-hydroxyl and/or acetylation of the 6′-amino group [8].

The three-dimensional structure of the APH(3′)-IIIa has recently been solved to 2.2 Å and been shown to have significant structural similarity to eukaryotic serine/threonine (Ser/Thr) and tyrosine (Tyr) protein kinases (EPK) despite a lack of significant amino-acid sequence homology (< 10% similarity) [9]. The recently determined structure of the type IIβ phosphatidylinositol phosphate kinase also demonstrates overall similarity with EPK and APH [10], so

Table 1

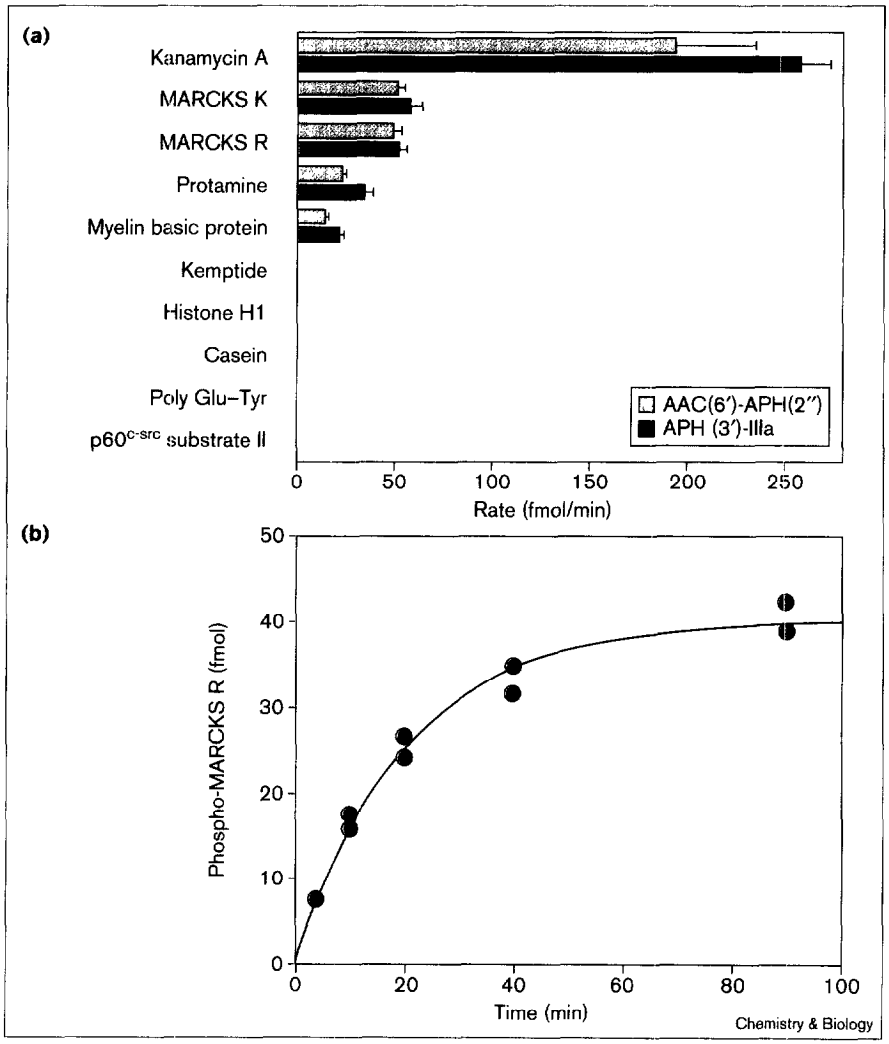
Protein kinase substrates.

Substrate	Sequence	Associated kinase
MARCKS K	Ac-FKKSFKL-NH_2	PKC
MARCKS R	Ac-FRRSFRL-NH_2	PKC
Kemptide	LRRASLG	PKA
Casein		Casein kinases
Histone H1		PKA, PKC, PKG
Protamine		PKC
MBP		PKA, PKC, MAP kinases
poly(Glu,Tyr) 4:1		Csk
$p60^{c\text{-}src}$ Substrate II	Ac-IYGEF-NH_2	c-Src

together these enzymes form a larger structurally related kinase family. Apart from overall three-dimensional structural similarity, the APHs also share a short amino-acid sequence with EPKs. The sequence (His(Gly/Asn)Asp–X_{3-4}–Asn (H(G/N)DX_{3-4}N; where X is any amino acid) from the APHs is homologous in both structure and function to the sequence (H/Y)RDX_4N from EPK [9]. The conserved aspartate (Asp190, APH(3′)-IIIa numbering), when replaced by an alanine by site-directed mutagenesis, shows dramatic reduction in enzymatic activity [9], an observation that parallels results obtained for various EPKs [11–14]. These mutagenesis studies, and the appropriate positioning of Asp190 in the active site, implicate this residue as a potential active-site base required for substrate hydroxyl deprotonation and therefore as a residue assisting in phosphoryl transfer.

The similarities in both the structure and mechanism of APH(3′)-IIIa and EPK have been subsequently investigated through the use of specific EPK inhibitors, including flavanoids and isoquinolinesulfonamides [15]. The latter group are effective competitive inhibitors of ATP and non-competitive inhibitors of aminoglycoside substrates of both

Figure 1



Protein phosphorylation by aminoglycoside phosphotransferases APH(3′)-IIIa and AAC(6′)-APH(2″). Potential substrates (50 μM) were incubated with purified enzymes for an appropriate amount of time and analyzed as described in the Materials and methods section. The rate of reaction was determined by fitting to a first-order rate equation. **(a)** Specificity of substrate phosphorylation. **(b)** Example of rate determination for the AAC(6′)-APH(2″)-catalyzed phosphorylation of the MARCKS R peptide.

APH(3′)-IIIa and AAC(6′)-APH(2″) with K_i values < 100 μM for the best derivatives. APHs and EPKs therefore share a similar overall three-dimensional structure, chemical mechanism of phosphoryl transfer and sensitivity to inhibitors.

To further investigate and establish the functional and mechanistic relationship between APHs and EPKs we report here the ability of two APH enzymes to phosphorylate known substrates of EPKs. The results demonstrate that APHs can act as serine protein kinases.

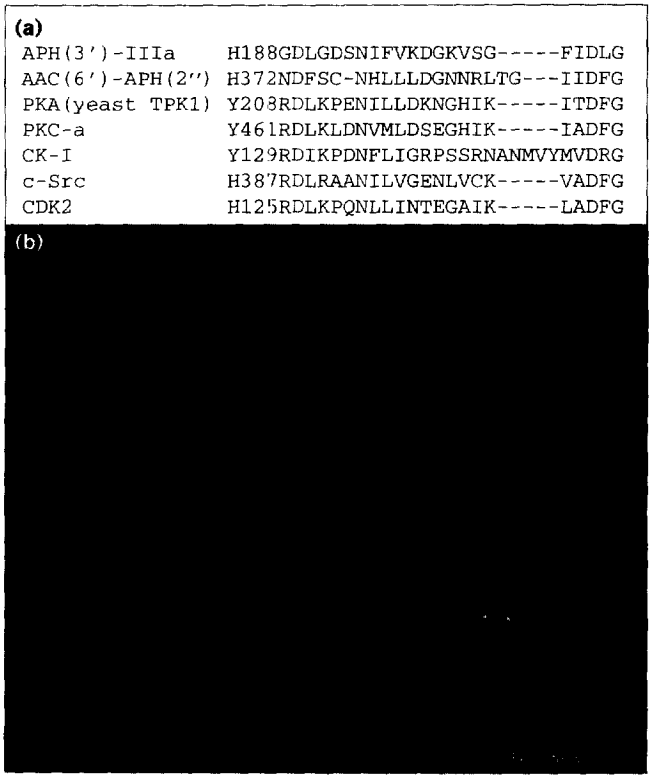
Results and discussion

Protein kinase activity of APHs

Previous analyses have indicated that APH(3′)-IIIa shares three-dimensional structure, chemical mechanism and sensitivity to inhibitors with EPKs. Additionally, the bifunctional enzyme AAC(6′)-APH(2″), which shows minimal sequence homology with APH(3′)-IIIa (10% identity, 21% similarity) and for which a three-dimensional structure is unknown at present, was also demonstrated to be sensitive to EPK inhibitors [15]. The structural and functional similarities between APHs and EPKs suggested the possibility that APHs could have the capacity to catalyze phosphoryl transfer to an EPK substrate. We therefore examined various known protein and peptide EPK substrates to determine if APHs could act as protein kinases. Our choices of EPK substrates were selected to investigate overall substrate diversity as well as specificity, that is Tyr or Ser/Thr phosphorylation (Table 1). Ser/Thr protein kinase specific substrates included MARCKS K and MARCKS R, derivatives of myristolated alanine-rich C-kinase substrate (MARCKS) protein [16], and protamine, which are all protein kinase C (PKC) substrates [17,18], casein, a substrate of casein kinase I and II [19], histone H1, a substrate of protein kinase A (PKA), PKC and protein kinase G [20], Kemptide, a PKA substrate [21], and myelin basic protein (MBP), a substrate of various protein kinases including PKA [22], PKC [23] and mitogen-activated protein kinases (p38, p42 and p44 MAP kinases) [23,24]. Two protein Tyr kinase substrates were also analyzed, poly(Glu,Tyr) (4:1), a synthetic peptide substrate for various tyrosine kinases (e.g. csk [14]), and $p60^{c\text{-}src}$ substrate II, a substrate of c-src kinase [25]. These peptides and proteins represent a broad spectrum of protein kinase activities that subsequently give a specific profile to APH protein kinase activities.

Several of the Ser/Thr kinase substrates, including the MARCKS peptides, protamine and MBP, were phosphorylated by both APH(3′)-IIIa and AAC(6′)-APH(2″), whereas Kemptide, histone H1 and casein were not substrates (Figure 1). The tyrosine kinase specific substrates, poly(Glu,Tyr) (4:1) or $p60^{c\text{-}src}$ substrate II were not substrates of either APH(3′)-IIIa or AAC(6′)-APH(2″).

Figure 2



Active-site region of protein and aminoglycoside kinases. **(a)** Sequence alignment of aminoglycoside and protein kinases highlighting conserved residues including Asp190 (green, APH(3′)-IIIa numbering), the putative active-site base, and the Mg^{2+} ligands, Asn195 (pink) and Asp208 (red). Genbank accession numbers: APH(3′)-IIIa, V01547; AAC(6′)-APH(2″), M13771; TPK1, M17072; Human PKCα, S09496; *Schizosaccharomyces pombe* CK-1, U06930, Human $pp60^{c\text{-}src}$, K03218; Human CDK2, X61622. **(b)** Close up of the APH(3′)-IIIa active site displaying ADP (orange) Mg^{2+} (cyan), the ADP coordinating residue Lys44 (light green), Asp190 (dark green), Asn195 (magenta), Asp208 (red). Gly189 is white. The structure was drawn using the programs MOLSCRIPT [38] and RASTER 3D [39].

Phosphorylation of the substrate MBP was also investigated using specific APH site mutants with impaired aminoglycoside kinase activity in an effort to confirm that protein phosphorylation was not due to contamination in either the enzyme or the substrate preparations. The APH(3′)-IIIa Asp190→Ala and Asp→208Ala mutants were selected on the basis of the known geometry of the active site and precedent in the EPK literature (Figure 2). Asp190 is therefore predicted to be involved in phosphoryl transfer and has a dramatically lower k_{cat} (> 500-fold) for aminoglycoside substrates when converted to alanine [9]. We also prepared the Asp208→Ala mutant as this residue co-ordinates Mg^{2+} in the APH(3′)-IIIa–ADP complex [9]. The analogous residue in yeast PKA (Asp228) has been mutated to alanine and lacks any detectable kinase activity, whereas the Asp210→Ala mutant, which is equivalent to APH(3′) Asp190, retained

Table 2

Kinetic parameters for mutant APHs.

Mutant APH	Substrate	$k_{cat}(s^{-1})$	$K_m(\Delta M)$	$k_{cat}/K_m(M^{-1}s^{-1})$	Ratio of kinetic parameters to wild-type enzyme* Δk_{cat}	ΔK_m	$\Delta k_{cat}/K_m$
APH(3′)-IIIa Gly189→Arg†	ATP	0.52 ± 0.02	320 ± 34	1.6×10^3	0.33	111.6	0.025
	Kanamycin A	0.10 ± 0.01	1050 ± 310	9.4×10^1	0.06	83.3	0.007
	Kanamycin B	0.60 ± 0.02	89.6 ± 7.0	6.6×10^3	0.17	4.60	0.036
	Neomycin C	0.15 ± 0.01	32.3 ± 4.5	4.8×10^3	0.07	4.2	0.018
	Ribostamycin	0.09 ± 0.005	91.3 ± 14.3	9.9×10^2	0.05	9.8	0.005
APH(3′)-IIIa Asp208→Ala	Kanamycin A	No activity detected‡					
AAC(6′)–APH(2″) Asp374→Ala	Kanamycin A	≤ 0.0019			0.004§		

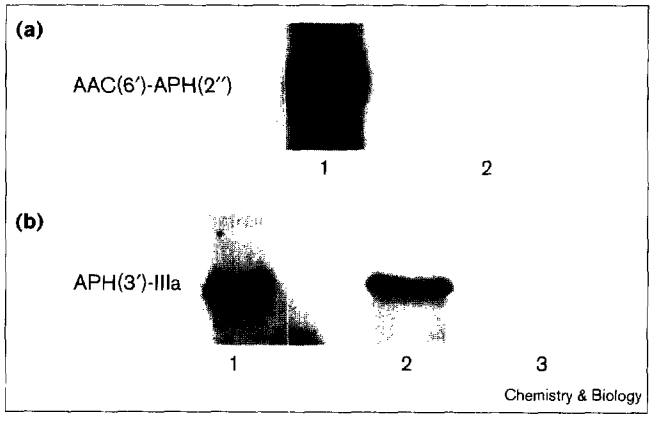
*Kinetic parameters for the wild-type enzyme were from [5]. †Other aminoglycosides that were found not to be phosphorylated by the enzyme were amikacin, butirosin, isepamicin, lividomycin and paromomycin. ‡Activity was not above background at 1 mM ATP, 0.1 mM kanamycin A. §Wild-type k_{cat} is 0.42 s^{-1} (D.M.D., G.D.W., unpublished observations).

only 0.4% of the wild-type activity, consistent with important roles for these residues in catalysis [12]. APH(3′)-IIIa Asp208→Ala lacks any detectable aminoglycoside kinase activity, as measured by ADP release coupled to pyruvate kinase and lactate dehydrogenase (Table 2). Phosphorylation of MBP by these mutant APH(3′)-IIIa enzymes was determined following a 4 h incubation (Figure 3). The Asp190→Ala mutant was only 52% as active as the wild-type enzyme, whereas the Asp208→Ala mutant was completely inactive as assessed by scintillation counting of excised phospho-MBP bands; protein phosphorylation is therefore due to the action of APH(3′)-IIIa and not due to contaminating protein kinase activity in either the substrate or the enzyme preparations. Additionally, the substrate MBP was boiled prior to addition to the reaction mix to inactivate any contaminating kinases, and this had no effect on phosphorylation in the presence of APHs. Similarly, the Asp374 residue of AAC(6′)-APH(2″) is equivalent, by sequence alignment, to Asp190 of APH(3′)-IIIa (Figure 2a). This enzyme has a greater than 200-fold impaired k_{cat} for aminoglycoside kinase activity using the ADP-release assay (Table 2) and also showed significantly diminished MPB phosphorylation capacity (6.7% of wild-type, Figure 3a) as expected. The fact that some protein phosphorylation activity was nonetheless detected in the Asp190 and Asp374 mutants reflects the longer incubation times and higher substrate concentrations used in this radioactive assay than in the spectrophotometric ADP release assay used to detect aminoglycoside phosphorylation.

The peptides and proteins that were phosphorylated by APHs have in common overall positive charge: pI > 10 for MARCKS peptides, MBP and protamine. Acidic peptides and proteins were not phosphorylated. The basis for discrimination, however, is not exclusively due to charge as histone H1 (pI > 10) and the peptide Kemptide with overall charge of +2 at pH 7.5 are not substrates. Nonetheless, the observed specificity is consistent with the fact that the substrate-binding pocket for APH(3′)-IIIa (and by implication AAC(6′)-APH(2″)) has an overall negative charge [9]. Under the conditions used to detect activity with APHs, the Ser/Thr kinase substrates were phosphorylated by either PKC or casein kinase-I as appropriate.

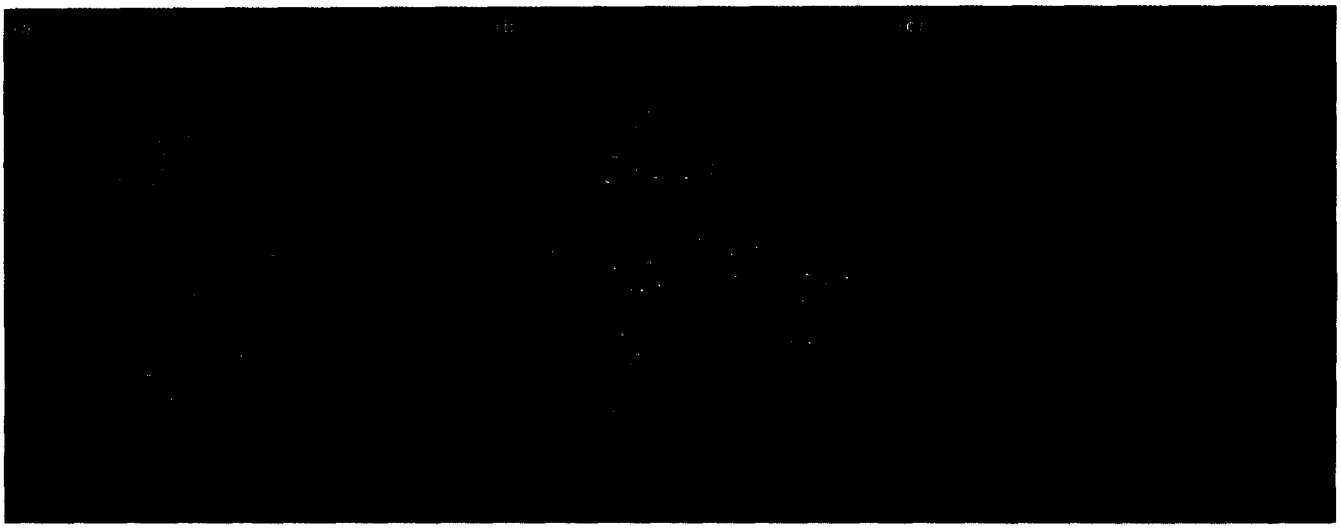
These results are all the more surprising when placed in the context of the three-dimensional structure of APH(3′)-IIIa and EPKs. Although the overall fold of APH(3′)-IIIa

Figure 3



Phosphorylation of MBP by APH(3′)-IIIa and AAC(6′)-APH(2″). MBP was phosphorylated with [γ^{32}P]-ATP by **(a)** AAC(6′)-APH(2″) or **(b)** APH(3′)-IIIa, separated on a 15% SDS–polyacrylamide gel and analyzed by autoradiography. Bovine MBP has a molecular weight of 18.3 kDa, but when phosphorylated, migrates at/or around 23 kDa. (a) Lane 1, wild-type AAC(6′)-APH(2″); lane 2, AAC(6′)-APH(2″) Asp374→Asn. (b) Lane 1, wild-type APH(3′)-IIIa; lane 2, APH(3′)-IIIa Asp190→Ala; lane 3, APH(3′)-IIIa Asp208→Ala. Similar results were found with native MBP and with MBP which was first boiled to inactivate any contaminating kinases.

Figure 4



Three-dimensional structures of APH(3′)-IIIa and casein kinase-1. Comparison of the three-dimensional structures of **(a)** APH(3′)-IIIa bound to ADP and **(b)** casein kinase-1 bound to ATP (PDB ID code 1CSN). The nucleotide cosubstrates are indicated in green and the Mg^{2+} ions are in magenta. The large cleft in casein kinase-1, which provides access to the active site for the protein and peptide substrates, is blocked in part in APH(3′)-IIIa by two helices between residues 135 and 178 shown in orange in the APH(3′)-IIIa structure. **(c)** Ribbon drawing of APH(3′)-IIIa. A loop region between residues 49–56 that lines the second substrate binding region of the active site is white. Structures (a) and (b) were drawn with the program RasMol v 2.6 [40], whereas (c) was drawn with MOLSCRIPT and RASTER 3D [39].

and EPKs is similar, a significant difference lies in the accessibility of the active site (Figure 4). In EPKs, such as casein kinase-1, the peptide/protein-binding site is open, whereas in APH(3′)-IIIa access is limited by several structural factors. Chief among these is an additional element in the carboxy-terminal domain consisting of two α helices that are positioned to provide a steric block to access by large substrates (Figure 4). In addition, a loop region contributed by the amino-terminal domain also serves to block access (Figure 4b). Attempts to delete the two α helices in the carboxy-terminal domain of APH(3′)-IIIa have so far yielded only insoluble and inactive protein (G.A.M. and G.D.W, unpublished observations). Nevertheless, the fact that some peptides and proteins are phosphorylated by APHs indicates the generous capacity of the active site.

The phosphorylation of MBP was further investigated to assess the rate and specificity of reaction. We initially attempted to determine V_{max} and K_m for these substrates without success, as the quantities of peptide required for enzyme saturation were incompatible with the assay. We therefore turned to a measure of k_{cat}/K_m using a sub-K_m concentration (10 μM) of MBP with equimolar enzyme. Under these conditions, the Michaelis–Menten equation simplifies to:

$$v = [S][E_{tot}]k_{cat}/K_m \quad (1)$$

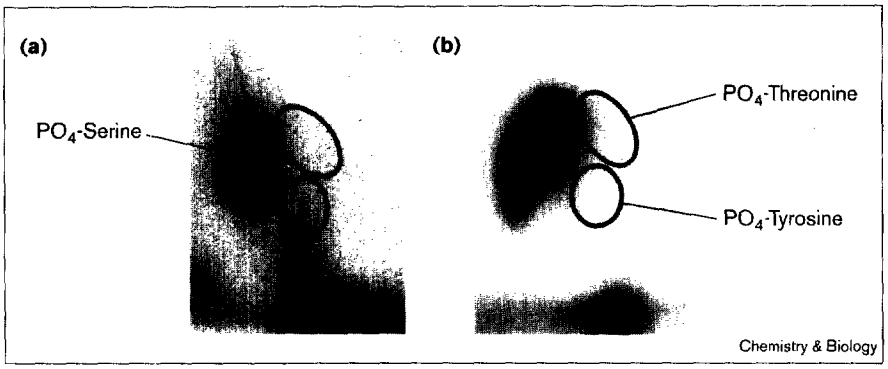
The values for k_{cat}/K_m for MBP were determined to be $4.5 \times 10^2\ M^{-1}s^{-1}$ for APH(3′)-IIIa and $8.0 \times 10^2\ M^{-1}s^{-1}$ for AAC(6′)-APH(2″). These catalytic efficiencies are 50- and 75-fold lower respectively than those obtained for kanamycin A using the phosphocellulose-binding assay under similar reaction conditions. The k_{cat}/K_m for PKC-catalyzed MBP phosphorylation is on the order of $10^6\ M^{-1}s^{-1}$ [26], so APH protein phosphorylation is, as expected, not comparable in efficiency with a dedicated protein kinase, but nevertheless demonstrates notable activity.

To further define and characterize the site of phosphorylation, phosphoamino acid analysis was performed on MARCKS K, MBP and protamine phosphorylated by APH(3′) or AAC(6′)-APH(2″). Phosphorylation of substrates by both enzymes occurred exclusively on serine residues (Figure 5), so APHs are protein Ser kinases. The reciprocal reaction, that is phosphorylation of aminoglycosides by EPKs, was assayed using purified casein kinase I and PKC however no detectable phosphorylation of kanamycin A or neomycin C (1 mM) occurred over 60 min (detection limit 0.7 fmol).

Characterization of APH(3′)-IIIa Gly189→Arg

Alignment of EPKs and APHs reveals a common catalytic peptide: His–Z–Asp–X_{3-4}–Asn ($HZDX_{3-4}N$; where Z is Gly, Asn or Arg, and X is any amino acid; Figure 2a), where the conserved aspartate is required for catalysis, possibly acting as an active-site base, and the conserved asparagine is essential for Mg^{2+} binding. The histidine residue is not necessary for catalytic activity in APH(3′)-IIIa [27] and indeed can be a tyrosine residue in some

Figure 5



Phosphoamino acid analysis of MARCKS K phosphorylated by AAC(6′)-APH(2″) and PKC. MARCKS K was phosphorylated by **(a)** AAC(6′)-APH(2″) and **(b)** PKC, hydrolyzed under acidic conditions and separated by two-dimensional thin layer electrophoresis as described in the Materials and methods section. The relative positions of standard phosphoserine, phosphothreonine and phosphotyrosine are indicated. Phosphorylation of MARCKS K, MARCKS R and protamine by both APH(3′)-IIIa and AAC(6′)-APH(2″) occurred exclusively on serine residues.

protein kinases. In APHs, the second amino acid, Z, is almost exclusively a glycine residue, although it is asparagine in the bifunctional AAC(6′)-APH(2″). On the other hand, with very few exceptions, the Z residue is an arginine in EPKs. We therefore mutated the equivalent glycine in APH(3′)-IIIa (Gly189) to arginine in an effort to determine if this change could impact on the protein kinase activity of this enzyme. Surprisingly, mutation of this residue abolished protein kinase activity and substantially disrupted aminoglycoside phosphorylation and specificity (Table 2). The drop in activity generally comes from both an increase in K_m and a decrease in k_{cat}. This suggests that this point mutation has caused significant change in the geometry of the active site and demonstrates that although EPKs and APHs share significant three-dimensional homology, mechanism and, now, substrate specificity, there nonetheless exist significant differences between the enzymes that will require substantial effort to unravel.

Significance

The data presented here conclusively demonstrate that aminoglycoside phosphotransferase (APH) enzymes tested in this study have intrinsic protein kinase activity, and suggest that other APHs will also retain this property. Thus APHs and eukaryotic protein kinases (EPKs) share three-dimensional structural features, chemical mechanism of phosphoryl transfer, sensitivity to inhibitors, and the capacity to phosphorylate protein and peptide substrates. Such similarities are unlikely to be due to convergence and suggest a direct evolutionary relationship between these enzymes. Recent evidence has shown that Ser/Thr and even Tyr kinases are not exclusive to eukaryotes and that many bacteria harbor such signal transduction enzymes (reviewed in [28,29]). In particular, Ser/Thr kinases have been either cloned from, or implicated in secondary metabolism in, actinomycetes [30–34]. Members of this group of bacteria are also important producers of aminoglycoside antibiotics and harbor APH genes themselves [2]. Actinomycetes therefore provide a potential common point of origin for antibiotic resistance enzymes and protein kinases.

The biological relevance, if any, of the protein kinase activity of APHs is at present unknown. These results might, however, impact on both eukaryotic and prokaryotic signal transduction experiments performed in which aminoglycoside resistance provides a selectable marker. For example the *neo* (encoding APH(3′)-II) or *hyg* (encoding APH(4)-I) genes confer resistance to the aminoglycoside antibiotics G418 and hygromycin B, respectively, and are frequently used in eukaryotic gene expression experiments. Our observation that two related APHs can phosphorylate proteins, although this activity is decidedly not robust, should be considered during the experimental design.

Materials and methods

Chemicals and enzymes

Myelin-basic protein from bovine brain, Kemptide, casein, poly(Glu,Tyr) (4:1) and kanamycin were from Sigma. Histone H1 from calf thymus and $p60^{c-src}$ substrate II were from Calbiochem. Protamine and MARCKS R peptide were the kind gift of Richard Epand, Department of Biochemistry, McMaster University. MARCKSK peptide was prepared by R.E. Williams, Institute for Biological Sciences, National Research Council, Ottawa, Ont. APH(3′)-IIIa and site mutants were purified as described previously [5]. The details for the overexpression and purification of AAC(6′)-APH(2″) will be submitted for publication. Site mutants were prepared by the QuikChange site-directed mutagenesis kit (Stratagene) using primers 5′-GGGTCCAGCGCCACCCTTTTCTG-3′, 5′-CTTCTCCCAAGAGCA-ATAAA-GCCAC-3′ and 5′-GTTTATGCCATAATAATTTTAGTTGTAATC-A-3′, and their reverse complements for APH(3′)-IIIa Gly189→Arg, APH(3′)-IIIa Asp208→Ala, and AAC(6′)-APH(2″) Asp374→Asn respectively. Rat brain PKC preparation (a mixture of α,β and γ isoforms) was the kind gift of R. Epand, Department of Biochemistry, McMaster University. Casein kinase-I from *Schizosaccharomyces pombe* was purified from *E. coli* BL21/pT7II-ckiΔ298 (kind gift of Jeff Kuret, Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL) as described previously [35].

Aminoglycoside kinase assay

Phosphorylation of aminoglycosides was monitored by coupling the release of ADP to the reactions catalyzed by pyruvate kinase and lactate dehydrogenase or by phosphocellulose binding assay as described previously [5].

Protein kinase assays

Phosphocellulose binding assay. Phosphorylation of peptide and aminoglycoside substrates was monitored by three separate assays. A phosphocellulose binding assay was employed for substrates which are positively charged at neutral pH and generally amenable to this protocol [36]. The assay consisted of peptide substrate (50–100 μM final concentration for rate determinations and 600 μM for determination of linearity with addition of enzyme), or aminoglycoside substrate (50 μM final), purified AAC(6′)-APH(2″) (15–60 μg) or purified APH(3′)-IIIa (7.5–30 μg), 10 mM [γ^{32}P]-ATP (1.20×10^5 cpm/nmol), 50 mM Tris pH 8.0, 40 mM KCl, 10 mM $MgCl_2$, in a final volume of 10 μl. The reactions proceeded at room temperature and were terminated after 5 min to 6.5 h by application onto Whatman P-81 phosphocellulose paper, washed three consecutive times with water, air dried and placed in scintillation vials with 5 ml of scintillation fluid.

Glass microfibre filter assay. Substrates with a negative or neutral charge at neutral pH and or those that weren't amenable to trapping on phosphocellulose paper, were assayed using filtration on glass microfibre filters. The reactions were prepared as in the above procedure. At the appropriate times, samples were applied onto Whatman GF/C glass microfibre filters and suction-filtered through a Millipore vacuum filtration apparatus. The filters were washed several times with the reaction buffer, air dried and placed in scintillation vials with 5 ml of scintillation fluid.

p60$^{c\text{-}src}$ Substrate II activity assays. Assay mixtures were comprised of peptide (500 μg/ml), 10 mM [γ^{32}P]-ATP (1.20×10^5 cpm/nmol), 100 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$ and 40 mM KCl. Reactions were initiated by the addition of 10–20 μg of purified enzyme and incubations were allowed to proceed for 0–4 h at ambient temperature. Samples were analyzed by reverse phase HPLC using a Spherisorb-ODS2 C18 column (4 mm × 20 cm) at a flow rate of 0.5 ml/min. Reactions were diluted fivefold to a final volume of 100 μl and applied to the column in a solution of 0.1% trifluoroacetic acid. Peptides were eluted by a gradient of 0.07% trifluoroacetic acid in acetonitrile (CH_3CN) (B). The gradient consisted of 10 minutes 0% B, 0 to 40% B over 50 min, a 10 min hold at 40% B followed by 40 to 90% B over 1 min. Eluent was monitored by simultaneously monitoring wavelengths from 210 to 300 nm using a photodiode array detector. Fractions (0.5 ml) were collected and analyzed by scintillation counting.

Phosphorylation of histone H1 and MBP. Incubations consisted of 0.4 mg/ml histone H1 or MBP and 10 mM [γ^{32}P]-ATP (1.20×10^5 cpm/nmol) in 50 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 40 mM KCl. The assays were initiated by the addition of 10–20 μg of either APH(3′)-IIIa or AAC(6′)-APH(2″) and allowed to progress for 4 h at ambient temperature. The samples were quenched by the addition of EDTA to a final concentration of 35 mM followed by an equal volume of 2 × SDS loading buffer (100 mM Tris-HCl pH 8.0, 2 mM EDTA, 10% glycerol, 4% SDS) and separated on a 15% SDS-polyacrylamide gel. Phosphorylation of the substrates was monitored by autoradiography of the dried gel.

Protein kinase phosphorylation of kanamycin. Two protein kinases, casein kinase I and PKC, were assayed by phosphocellulose filter binding assay for their ability to modify the aminoglycoside kanamycin. Assay conditions were identical to those described above with the exception that 25 μg of either partially purified casein kinase I or PKC were used instead of an APH. For PKC activity assays large unilamellar vesicles composed of phosphotidylserine and phosphatidylcholine were added to a final concentration of 100 μM. The reactions were allowed to proceed for 1–4 h and then applied to Whatman P81 phosphocellulose paper and analyzed as above.

Analysis of rate data. Plots of the amount of phosphate incorporated into substrate proteins or peptides as a function of time were fit by nonlinear least squares method to a first order rate equation:

$$v = k\,[S] \qquad (2)$$

Phosphoamino acid analysis of phosphorylated products

Peptides (MARCKS R, MARCKS K and protamine) were first subjected to an *in vitro* kinase reaction that consisted of 15 μg of AAC(6′)-APH(2″), 32 μg of peptide, [γ^{32}P]ATP to 10 mM final (1.20×10^5 cpm/nmol) in 20 μl of 50 mM Tris pH 7.5, 40 mM KCl and 10 mM $MgCl_2$. Reactions proceeded for 2 h. Separation of the phosphorylated peptide from ATP and enzyme was accomplished by binding to Whatmann P-81 phosphocellulose paper followed by three consecutive washes and drying.

The dried 1 cm^2 piece of phosphocellulose was placed in a screw cap Eppendorf tube with 1 ml of 6N HCl and incubated in a 110°C oven for 90 min. The hydrolysate was evaporated under vacuum and the sample was then dissolved in 10 μl of water.

Separation and identification of phosphoamino acids was performed by two-dimensional thin layer electrophoresis [37]. A 5 μl volume of the sample was applied in 0.5 μl aliquots followed by drying on an origin of a 20 cm × 20 cm × 100 μm glass-backed cellulose thin layer chromatography plate. Non-radioactive standard phosphoserine, phosphothreonine and phosphotyrosine were also applied at the origin. The buffer for the first dimension of electrophoresis consisted of 50 ml of 88% formic acid (0.58 M final concentration), 156 ml of glacial acetic acid (1.36 M final concentration) and 1794 ml water all at pH 1.9. The plate was the electrophoresed for 20 min at 1.5 kV in a Hunter Thin Layer Peptide Mapping System Model# HTLE-7000. Following electrophoresis the plate was removed and air dried.

The second dimension buffer consisted of 100 ml of glacial acetic acid (0.87 M final concentration), 10 ml of pyridine (0.5% (v/v) final concentration, 10 ml of 100 mM EDTA (0.5 mM final concentration) and 1880 ml of water all at pH 3.5. The plate was rotated 90° to the first dimension electrophoresis and re-electrophoresed for 16 min. at 1.3 kV. The plate was dried, sprayed with 0.25% ninhydrin in ethanol and heated to visualize the standards. Autoradiography was then used to visualize the samples.

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