Mechanism of aminoglycoside 3'-phosphotransferase type IIIa: His188 is not a phosphate-accepting residue

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Background: The enzyme aminoglycoside 3'-phosphotransferase Type IIIa (APH(3')-IIIa), confers resistance to many aminoglycoside antibiotics by regiospecific phosphorylation of their hydroxyl groups. The chemical mechanism of phosphoryl transfer is unknown. Based on sequence homology, it has been suggested that a conserved His residue, His188, could be phosphorylated by ATP, and this phospho-His would transfer the phosphate to the incoming aminoglycoside. We have used chemical modification, site-directed mutagenesis and positional isotope exchange methods to probe the mechanism of phosphoryl transfer by APH(3')-IIIa.

Results: Chemical modification by diethylpyrocarbonate implicated His in aminoglycoside phosphorylation by APH(3')-IIIa. We prepared His → Ala mutants of all four His residues in APH(3')-IIIa and found minimal effects of the mutations on the steady-state phosphorylation of several aminoglycosides. One of these mutants, His188Ala, was largely insoluble when compared to the wild-type enzyme. Positional isotope exchange experiments using γ-[^{18}O]-ATP did not support a double-displacement mechanism.

Conclusions: His residues are not required for aminoglycoside phosphorylation by APH(3')-IIIa. The conserved His188 is thus not a phosphate accepting residue but does seem to be important for proper enzyme folding. Positional isotope exchange experiments are consistent with direct attack of the aminoglycoside hydroxyl group on the γ-phosphate of ATP.

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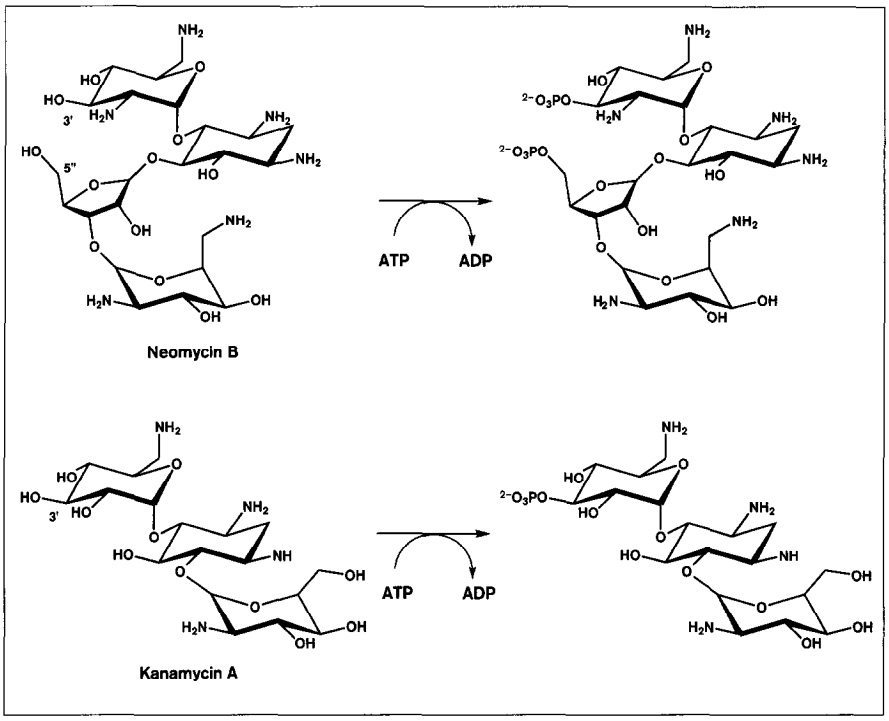
Introduction

Hospital-acquired infections have become a particularly serious problem because of the widespread dissemination of antibiotic resistance genes. The Gram positive cocci of the genera *Enterococcus* and *Staphylococcus* are among the more prevalent causes of these infections [1]. Enterococcal infections are treated with a combination of aminoglycoside and β-lactam antibiotics, or with the glycopeptide vancomycin [2]. These infections have become increasingly difficult to treat because of the selection of large numbers of antibiotic resistance determinants [3]. This increase in antibiotic-resistant organisms has brought into question the future usefulness of aminoglycosides and other antibiotics [4,5].

In general, aminoglycoside antibiotics are composed of a central 2-deoxystreptamine aminocyclitol ring which is linked to various aminosugars via glycosyl bonds (e.g., Fig. 1). Bacterial resistance to the aminoglycosides occurs predominantly through a variety of enzyme-catalyzed processes [6]. These mechanisms involve the covalent modification of aminoglycosides by *O*-phosphorylation, *N*-acetylation or *O*-adenylation. These modifications are catalyzed by a superfamily of transferases, comprising greater than fifty individual enzymes [7,8]. Species of both the *Enterococcus* and *Staphylococcus* genera can harbor specific aminoglycoside-modifying isozymes, which circumvent the bactericidal effects of these antibiotics [9]. One of these enzymes, the 3'-aminoglycoside phosphotransferase type IIIa (APH(3')-IIIa), confers resistance to a broad spectrum of aminoglycosides including the clinically important drug amikacin [10,11].

APH(3')-IIIa catalyzes the transfer of the γ-phosphate of ATP to the 3' hydroxyl group of 4,6-disubstituted deoxystreptamine aminoglycosides and to both the 3'- and 5"-hydroxyls of 4,5-disubstituted deoxystreptamine aminoglycosides, such as neomycin, that possess available hydroxyl groups (Fig. 1) [12]. Six other classes of APH(3')s have been identified and their protein sequences have significant homology in the carboxy-terminal region [8]. Three conserved motifs have been identified (Fig. 2) and, although their function is not known, Martin *et al.*) [13] have speculated that, based on homology searches of protein databases, motif 2 is part of the nucleotide binding site, motif 3 is involved in aminoglycoside binding and motif 1 is directly involved in catalysis. Specifically, they postulated that the strictly conserved histidine in APH(3') enzymes (His188 in APH(3')-IIIa) may act as a phosphate-accepting residue, mediating the transfer of

Figure 1

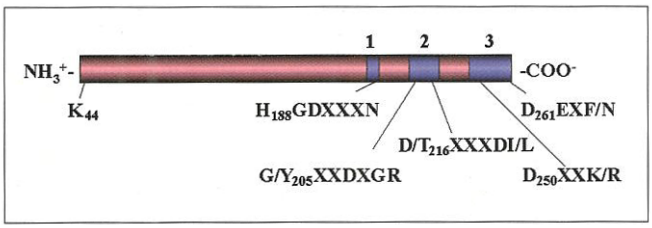


APH(3')-IIIa catalyzes the regiospecific transfer of the γ-phosphate of ATP to an aminoglycoside hydroxyl group. Two examples are shown.

the γ-phosphate of ATP to an aminoglycoside hydroxyl group via a phospho-enzyme intermediate (Fig. 3a) [13]. We have not found any steady-state kinetic evidence for a phospho-enzyme intermediate with APH(3')-IIIa [14,15]. Thus, an equally plausible mechanism of phosphate transfer would be by the direct in-line transfer of the terminal phosphate of ATP to a specific aminoglycoside hydroxyl group (Fig. 3b) [16,17]. A dissociative mechanism in which a hyper-electrophilic metaphosphate anion is generated is theoretically possible, but would be unprecedented. All known enzymatic phosphoryl transfers occur by associative mechanisms [17].

Site-directed mutagenesis of His188 has been reported for the APH(3')-II from *Tn*5. Substitution of His188 with residues, such as Ser and Tyr, or Leu [18], that are potential phosphate acceptors (but very poor phosphate donors), or by Gln [19] demonstrated the importance of this residue for conferring aminoglycoside resistance; for cells harboring a mutant enzyme, a dramatic decrease in minimal inhibitory concentrations for a number of aminoglycosides was observed. Assays of crude cell-free extracts on the His188Gln mutant revealed a two-fold increase in the K_M for ATP and a 35-fold decrease in relative activity, but the enzyme was not purified [19].

Figure 2

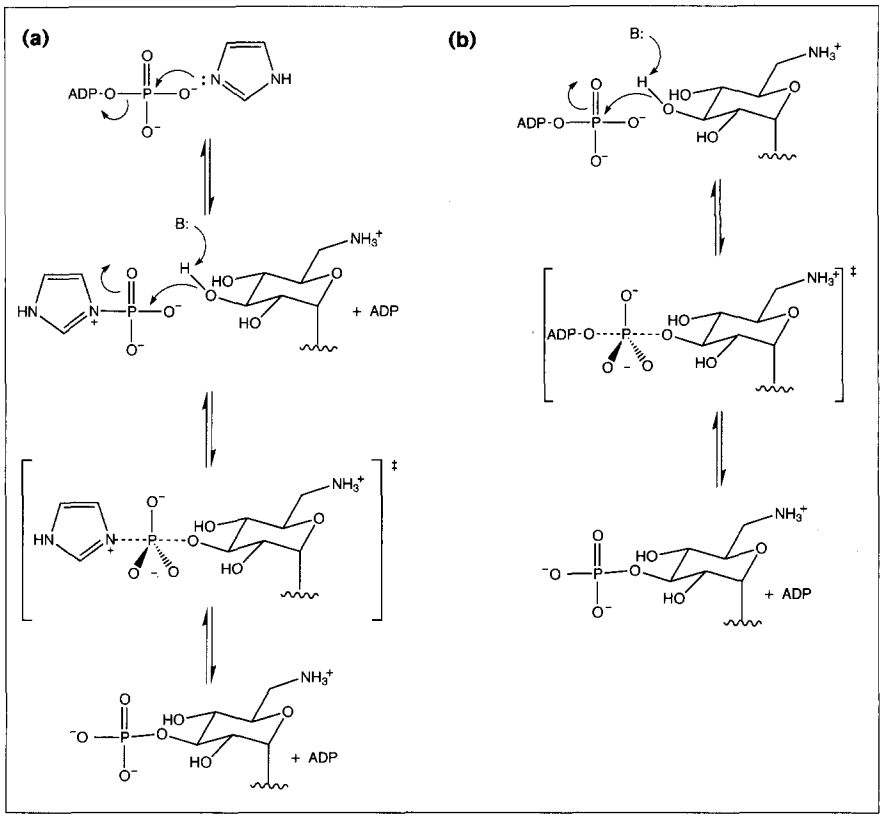


Conserved motifs (1,2,3) in APH(3') sequences. His188 is found in motif 1. Lys44 can be labeled with *p*-fluorosulfonylbenzoyl adenosine [38], and thus contacts ATP. The single-letter code for amino acids is used.

To understand the mechanism of phosphoryl transfer for the rational design of inhibitors and/or new antibiotics, we have undertaken to delineate the chemical mechanism of phosphoryl transfer. The question of whether phosphate transfer occurs through a phospho-enzyme intermediate or by a direct in-line attack is complicated by the fact that the kinetic mechanism of APH(3')IIIa is Theorell–Chance [14]. In this version of an ordered BiBi mechanism, ATP binds to the enzyme first and the aminoglycoside second; this is followed by release of the phospho-aminoglycoside and ADP. In such a mechanism the ternary complex is transparent in the steady state because product release is completely rate limiting, and as such the existence of a phospho-enzyme intermediate cannot be discounted. To discern between double-displacement (phospho-enzyme) and direct-attack associative mechanisms, and to elucidate the possible role of His188 in catalysis, we have approached the problem in two ways. First, we examined

Figure 3

Possible mechanisms of phosphate transfer catalyzed by APH(3')-IIIa. The reaction may proceed **(a)** by a double-displacement mechanism in which a phospho-His intermediate is formed or **(b)** by direct in-line transfer of the terminal phosphate of ATP to a specific aminoglycoside hydroxyl group.



the roles of the His residues in APH(3')-IIIa by chemical modification and site-directed mutagenesis. Second, we addressed the question of the route of phosphoryl transfer (Fig. 3a versus 3b) by the positional isotope exchange (PIX) technique of Midlefort and Rose [20,21]. These experiments provide evidence that neither His188 nor any other His residue in the enzyme is required for catalysis and that the most likely mechanism is direct attack of the aminoglycoside hydroxyl group on the γ-phosphate of ATP.

Results

Inactivation of APH(3')-IIIa by chemical modifying agents

We initially explored the roles of several different amino acid residues in catalysis by a survey of the susceptibility of APH(3')-IIIa to various chemical modifying agents (Table 1). The enzyme showed broad sensitivity to compounds modifying carboxyl and thiol groups as well as Tyr and Arg residues. The susceptibility to diethylpyrocarbonate (DEPC) suggested a possible role for His residues in catalysis [22]. Inactivation by DEPC was largely overcome by the subsequent addition of hydroxylamine (not shown). This result, and the observation that DEPC treatment caused an increase in absorbance at 242 nm but not at 278 nm, supported the notion that His is modified rather than Tyr, which is also a possible target for this agent [22]. Rates of inactivation were best fit to a double exponential decay model, suggesting that more than one residue was modified and that these modifications had different effects on the enzymatic rate (Table 2). This was confirmed by stoichiometric measurements, which indicated that approximately three His residues were modified (Table 2).

Table 1

Chemical modification of APH(3')-IIIa.

Reagent	Target	Concentration (mM)	Activity (%)
Control, pH 7.5	–	–	100
N-Ethylmaleimide	Cys	0.5	47
p-Chloromercuribenzoic acid	Cys	0.5	3
Diethylpyrocarbonate	His, Tyr, Cys	10	2
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide	C-term COO^-, Asp, Glu	2.5	23
Tetranitromethane	Tyr	2.5	1.8
Control, pH 8.0	–	–	100
Phenylglyoxal	Arg	2.5	10

Table 2

Inactivation of APH(3')-IIIa by DEPC.

Enzyme	Stoichiometry	Rates of inactivation	
		Rate 1	Rate 2
Wild type	3.4	0.17 ± 0.054	0.010 ± 0.011
His78Ala	2.3	0.49 ± 0.156	−0.0026 ± 0.0063
His82Ala	2.9	2.12 ± 1.75	0.032 ± .0256
His123Ala	3.3	0.36 ± 0.12	0.0049 ± 0.0067
His188Ala	2.0	0.75 ± 0.047	0.0085 ± 0.0087

His→Ala mutagenesis of APH(3')-IIIa

There are four His residues in APH(3')-IIIa: His78, His82, His123 and His188. All four were individually mutated to Ala, and the mutant proteins were overexpressed in *Escherichia coli* and purified. The mutant enzymes were first characterized by steady-state kinetic methods (Table 3). None of the mutants demonstrated dramatic deviation from wild-type behavior, and V/K was essentially unchanged. Since His188 is absolutely conserved in all known APH(3') sequences and had been proposed to be important in catalysis, we determined the accurate molecular mass by electrospray mass spectrometry to ensure that the kinetic results were not due to contamination by native enzyme. The mass was determined to be 30 778 Da, demonstrating the integrity of the His188Ala mutant (predicted mass: 30 779 Da, less the amino-terminal Met). We did, however, observe that the yield of purified His188Ala protein was very poor (< 1 mg l^{-1} of cell culture compared to >50 mg for the wild-type enzyme). SDS-PAGE and Western analysis of the soluble and insoluble fractions following cell lysis revealed that the His188Ala mutant was highly expressed but was found mostly as an inactive, precipitated form (Fig. 4).

All four His mutant proteins were DEPC sensitive, and had similar inactivation kinetics (Table 2). The stoichiometries of inactivation were also similar, but suggest that His123 is not as readily modified as the others and thus is probably buried within the folded protein.

PIX Experiments

We performed PIX experiments with wild-type APH(3')-IIIa using ATP specifically enriched with ^{18}O-labeled phosphate in the γ-position (Fig. 5). The experiment was performed at saturating concentrations of γ-[^{18}O]-ATP, with either APH(3')-IIIa alone, or APH(3')-IIIa in the presence of tobramycin, a competitive inhibitor of APH(3')-IIIa that lacks the accepting 3'-hydroxyl group. The ^{31}P NMR spectra corresponding to the β and γ phosphate of ATP remain unchanged under all conditions tested (see Fig. 6). The formation of a transient phosphoenzyme intermediate would have resulted in the appearance of new peaks downfield from those corresponding to the γ phosphate and upfield from the β-phosphate peaks [23]. The lack of a PIX is inconsistent

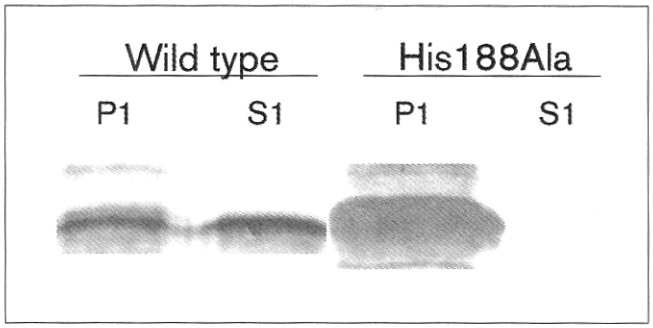
Table 3

Steady state kinetic parameters for wild type and mutant APH(3')-IIIa proteins.

	Wild Type[a]			His78Ala		
Substrate	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)
ATP	27.7 ± 3.7	1.76 ± 0.08	–	10.9 ± 1.8	0.66 ± 0.01	–
Kanamycin A	12.6 ± 2.6	1.79 ± 0.09	6.38 ± 1.67	5.1 ± 1.6	0.67 ± 0.04	1.68 ± 0.86
Neomycin B	7.72 ± 0.9	2.08 ± 0.07	2.65 ± 0.59	27.0 ± 5.1	2.46 ± 0.18	1.84 ± 0.58
Amikacin	245 ± 27	2.46 ± 0.11	ND	206 ±13	1.74 ± 0.04	–
	His82Ala			His123Ala		
	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)
ATP	12.17 ± 2.5	0.35 ± 0.01	–	11.6 ± 1.8	0.571 ± 0.02	–
Kanamycin A	2.44 ± 0.52	0.35 ± 0.01	6.7 ± 2.8	0.92 ± 0.2	0.49 ± 0.01	5.03 ± 2.82
Neomycin B	1.31 ± 0.20	0.69 ± 0.01	–	0.29 ± 0.13	0.83 ± 0.01	6.95 ± 3.13
Amikacin	93.1 ± 10	0.50 ± 0.01	–	110 ± 13	0.87 ± 0.03	–
	His188Ala					
	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)			
ATP	33.5 ± 5.9	3.75 ± 0.16	–			
Kanamycin A	17.4 ± 1.7	3.36 ± 0.07	–			
Neomycin B	3.2 ± 0.7	1.20 ± 0.05	2.74 ± 0.88			
Amikacin	184 ± 30	2.04 ± 0.09	–			

[a]Kinetic parameters for wild type APH(3')-IIIa are reproduced from [11].

Figure 4



His188Ala APH(3')-IIIa is expressed at high levels but is largely insoluble compared to the wild-type protein. The insoluble (P1) and soluble (S1) fractions from total cell lysates were separated by centrifugation. Proteins (2 μg) were applied to an SDS-polyacrylamide gel (15 %), electroblotted onto nitrocellulose and probed with rabbit anti-APH(3')-IIIa. Western blots were visualized by incubation with alkaline-phosphatase-conjugated donkey-anti-rabbit IgG and developed with 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt/nitroblue tetrazolium chloride. A faint band in the His188Ala S1 fraction can be seen in the original blot.

with the hypothesis that phosphate transfer is mediated by a phospho-enzyme intermediate.

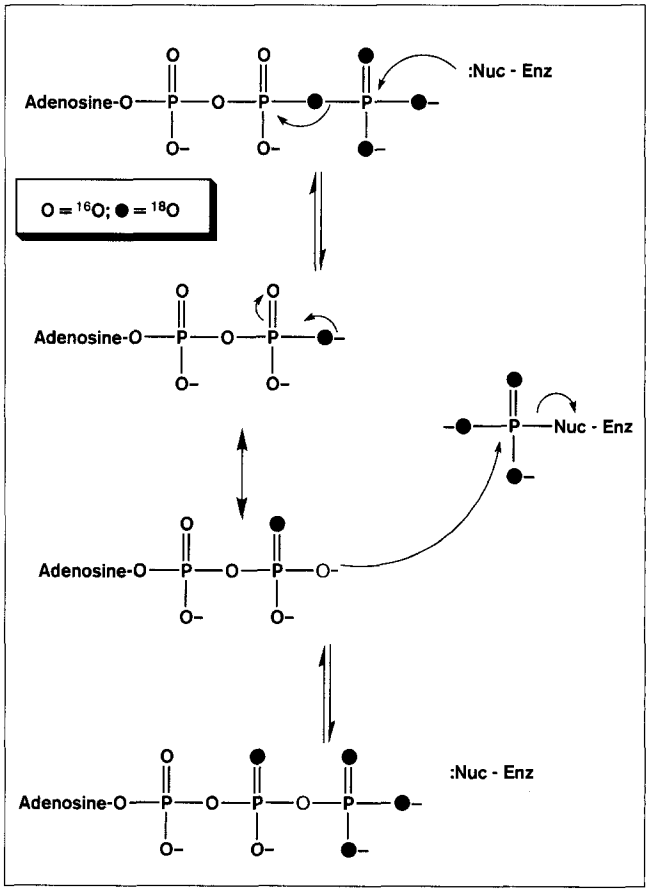
Discussion

The emergence of drug-resistant bacteria, especially multiply-resistant Enterococci and Staphylococci, is a major concern for health care [1,24]. As there is a shortage of new antibiotics to fight infection with these bacteria, the redesign of available antibiotics or the design of inhibitors of bacterial antibiotic-resistance mechanisms are likely to be important. Both of these methodologies will be facilitated by a thorough understanding of the resistance mechanisms involved. In the case of the aminoglycoside class of antibiotics, the high prevalence of enzymes that mediate resistance via phosphorylation of the drugs and the lack of detailed mechanistic data has led us to study APH(3')-IIIa [11]. The apparent paradox presented by the broad specificity of APH(3')-IIIa [11], in contrast to its high regiospecificity [12], suggests that it would be a good model for the study of an aminoglycoside phosphorylation.

Classical dead-end and product-inhibition analysis in the steady state, as well as supporting evidence in the form of isotope and viscosity effects, all confirm that APH(3')-IIIa operates by a Theorell–Chance kinetic mechanism [14,15]. In this mechanism, ATP binds first, the aminoglycoside then enters, is phosphorylated and then released, followed by rate-limiting dissociation of ADP. Although a ping-pong kinetic mechanism is generally diagnostic of a double-displacement (phospho-enzyme) mechanism, the kinetic mechanism of APH(3')-IIIa is nonetheless consistent with both direct nucleophilic attack of the aminoglycoside hydroxyl group on ATP and a phospho-enzyme double-displacement mechanism.

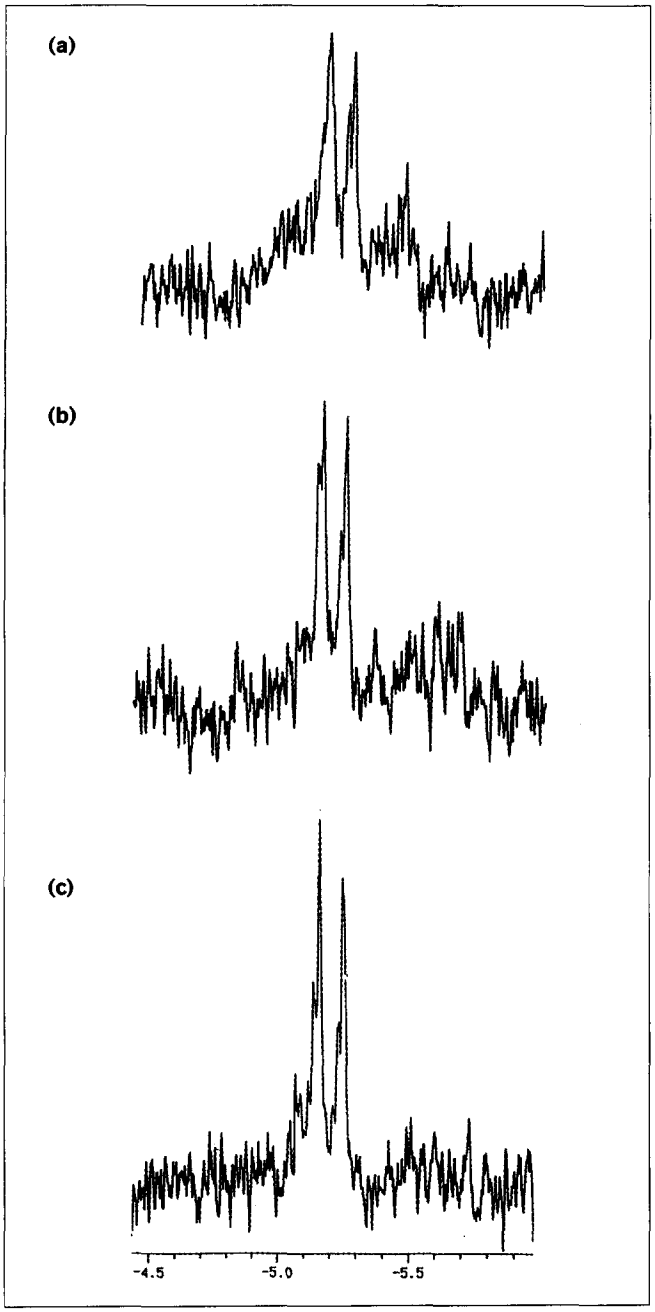
Although no direct evidence for either mechanism had been presented, the presence of an invariant His residue in all known APH(3') enzymes suggested the possibility that this residue participates in catalysis, perhaps as a phosphate-accepting residue [13]. This suggestion was proposed, in part, because the sequence His-Xaa-Asp is conserved in several nucleotide-binding proteins such as adenylate kinase, protein kinases and elongation factors [13]. This sequence similarity can now be reexamined in the light of many more amino-acid sequences and of X-ray crystal structures of some of the aforementioned proteins. For example, the His-Xaa-Asp triad [13], does not appear to have a role in nucleotide binding, based on the three-dimensional structures of adenylate kinase, EF-Tu and EF-G [25–27].

Figure 5



Positional isotope exchange by an enzyme using a double-displacement mechanism. In ^{31}P NMR of the complex of labeled ATP and enzyme, the formation of the transient phospho-enzyme intermediate would result in the appearance of new peaks downfield from those corresponding to the γ phosphate and upfield from the β-phosphate peak.

Figure 6



^{31}P NMR of γ-[^{18}O]-ATP complexed with APH(3')-IIIa indicates that the formation of a phospho-enzyme intermediate is not involved in the catalytic mechanism of the enzyme. The figure shows ^{31}P NMR with **(a)** standard γ-[^{18}O]-ATP, **(b)** γ-[^{18}O]-ATP + APH(3')-IIIa and **(c)** γ-[^{18}O]-ATP + APH(3')-IIIa + tobramycin (20 μM). APH(3')-IIIa (0.1 nmol) ± tobramycin was incubated for 15 min with γ-[^{18}O]-ATP (0.4 mM) at 37 °C. Approximately 40 % (158 nmol) of the available ATP was expected to have been turned over during incubation with APH(3')-IIIa. The linewidths of the sharpest peaks in the spectra are approximately 1.5 Hz. Based on a digital resolution of 0.47 Hz per point a minimum peak separation of 1.0 Hz is possible. The data was processed with a 1.0 Hz line broadening enabling the observation of peaks shifted downfield by 4.7 Hz, indicative of $^{18}O_3P^{16}O$, relative to the $P^{18}O_4$ resonance. No $^{18}O_2P^{16}O_2$ species was detected in the region approximately 8.0 Hz downfield of the $P^{18}O_4$.

Figure 7

APH(3')-IIIa	H188	GDLGDSN
cAPK	Y164	RDLKPEN
CDK2	H125	RDLKPQN
IRK	H1157	RDLAARN
ERK2	H147	RDLKPSN
CSK	H311	RDLAARN
SRC	H187	RDLRAAN
YES	H394	RDLRAAN
ABL	H361	RDLAARN

Partial alignment of conserved kinase sequences shows conserved His, Glu and Asn residues. The single-letter code for amino acids is used. Swiss-Pro database accession numbers are: APH(3')-IIIa, P00554; human cAMP-dependent protein kinase (cAPK), P22694; human cyclin-dependent protein kinase 2 (CDK2), P24941; human insulin receptor precursor (IRK), P06213, human MAP kinase ERK2, P28482; human CSK, P41240; human c-SRC, P12931; human c-YES, P07947; human c-ABL, P00519.

The motif 1 sequence His-Xaa-Asp-Xaa-Xaa-Xaa-Xaa-Asn was previously noted in a few eukaryotic protein kinases [13,28] and can now be identified in many different protein kinases (Fig 7). The crystal structures of the Ser/Thr cyclin-dependent kinase 2 (Cdk2) [29], the Ser/Thr MAP kinase Erk2 [30], and the tyrosine kinase domain of the human insulin receptor (IRK) [31] have recently been solved and shed some light on the role of this conserved sequence in protein kinases. The conserved His residue does not seem to have a direct role in catalysis. But, based on the structure and proposed mechanism of the cAMP-dependent protein kinase (cAPK), which lacks the conserved His, it has been suggested that the conserved Asp residue acts as a general base for deprotonation of the nucleophilic hydroxyl group and that the conserved Asn participates in an H-bond network required for Mg^{2+} coordination [32]. This sequence similarity suggests that the active site of APH(3')-IIIa may share many characteristics of the protein kinase structures.

As a first step towards determining the mechanism of APH(3')-IIIa catalysis, we have explored the role of His residues. Chemical inactivation experiments suggested a role for COO^- groups, Tyr, Arg, possibly Cys, and His. The sensitivity of APH(3')-IIIa to the His-modifying agent DEPC was complex, and rate and stoichiometry experiments clearly demonstrated that more than one His residue are modified. Substrate-protection experiments were attempted but showed variable results, possibly because of interactions with DEPC. Chemical modification experiments are, however, notoriously difficult to interpret, largely due to the indiscriminate nature of the reactive agents.

To effectively dissect the possible role(s) of the four His residues of APH(3')-IIIa in catalysis, we turned to site-directed mutagenesis. Mutagenesis of all four His residues individually to Ala proved highly informative. Neither His78 nor His82, which are not conserved among other APH(3') enzymes, were important for enzyme function. The His→Ala mutants were still DEPC sensitive and did not have dramatically perturbed steady-state kinetics. His123, which is conserved in all but the class VIa and VIIa 3'-aminoglycoside phosphotransferases [8], was also not critical for APH(3')-IIIa activity. His188 is conserved among all APH(3') enzymes, but again mutation to Ala had little effect on activity or DEPC sensitivity. There was, however, a dramatic effect on the recovery of active, soluble enzyme when His188 was replaced by Ala. This demonstrates that His188 is not required for catalysis, and is therefore not a phosphate accepting residue, but is probably critical for efficient protein folding. This result is also consistent with the homology to protein kinases where the conserved His residue does not appear to directly participate in catalysis.

Characterization of the His→Ala mutants unequivocally demonstrated that His residues are not important for APH(3')-IIIa activity. Thus, we could conclude that the catalytic mechanism does not involve a phospho-His intermediate, but the possibility still existed that a phospho-enzyme intermediate involving another residue, (e.g., phospho-Asp, Glu or Cys), was involved. We chose to explore this possibility by using the PIX methodology of Midlefort and Rose [20]. Incubation of the enzyme with γ-[^{18}O]-ATP followed by formation of a phospho-enzyme intermediate should permit the scrambling of ^{18}O from the β-γ-bridge to a non-bridge position two out of three times assuming the likely, ready exchange of oxygen between these positions (Fig. 5). This exchange is readily detectable by ^{31}P NMR [23]. No PIX was observed with APH(3')-IIIa, even when the enzyme was incubated with the 3'-deoxy aminoglycoside, tobramycin, to give a non-productive ternary complex. These results are consistent with direct in-line attack of the aminoglycoside nucleophilic OH on the γ-phosphate of ATP (Fig. 3b) and are inconsistent with formation of a phospho-enzyme intermediate.

Significance

We have shown that His residues do not have a role in APH(3')-IIIa catalysis and that the reason that His188 is conserved among all APH enzymes may be because it is required for proper protein folding. The fact that many protein kinases also share the HXDXXXXN motif, and that the conserved His residue is not important in the catalytic mechanism of these enzymes, raises the tantalizing possibility that APHs and protein kinases share a common mechanism and structure. This hypothesis can be tested through further site-directed mutagenesis studies and structural experiments. It is also tempting to speculate that a common ancestral hydroxyl-group kinase may have existed.

The PIX experiments support a direct-displacement mechanism for the phosphoryl transfer reaction. The mechanism does not require formation of a phospho-enzyme intermediate. Thus, analogs that mimic the pentacoordinate transition state shown in Figure 3b should be good inhibitors of the enzyme and could be therapeutically useful. There is no good reason that such compounds would have to be aminoglycosides themselves, and thus it may be possible to develop a new class of aminoglycoside-kinase inhibitors.

Materials and methods

Chemicals

Kanamycin A, neomycin B, amikacin, tobramycin and pyruvate kinase/lactate dehydrogenase were obtained from Sigma (St. Louis, MO). ATP was obtained from Boehringer Mannheim (Laval, Quebec, Canada). $H_2{}^{18}O$ (97.8 atom%) was obtained from Isotec (Miamisburg, OH). $KH_2P^{18}O_4$ was synthesized by the method of Risley and Van Etten [33]. [γ-^{18}O]-ATP was prepared by reaction of $KH_2P^{18}O_4$ with ADP-morpholidate as previously described [34].

Site-directed mutagenesis of His 78, 82, 123, and 188 to Ala

The His188 to Ala mutant was generated by Kunkel methodology [35] using the oligonucleotide 5'-CTCCCAGGTCGCCGGCGGAAAAGA-CGAGCTC-3'. The mutated gene was subcloned into pET-22b(+) (NovaGen, Madison, WI) using the restriction enzymes *Nde*I and *Hind*III to generate the expression construct pAPHH188A.

Mutagenic oligonucleotides 5'-CATGCCGTTCAAAGGCCAGGACCT-TTGG-3', 5'-GCTCCAGCCATCAGCCCGTTCAAAGTGC-3', and 5'-CGATATGTCGATCGAGGCAAAGAGCCTGATGC-3' were used to generate the His to Ala mutants 78, 82 and 123, respectively, by the megaprimer PCR mutagenesis method [36]. In all cases, a megaprimer was amplified from pETSACG1 using the mutagenic oligonucleotide and our 5' PCR primer [11] in the polymerase chain reaction (PCR). In subsequent PCR reactions mutagenic megaprimers were isolated and used to amplify the full length gene using our 3' PCR primer. The isolated fragments were digested with *Nde*I and *Hind*III and ligated into similarly cut pET-22b(+). Presence of the desired mutations in pETH78A and pETH82A constructs were confirmed by sequencing at The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Presence of the desired mutation in pETH123A constructs was confirmed by the incorporation of a silent *Pvu*I site in the mutagenic oligonucleotide. All confirmed constructs were subsequently sequenced in their entirety to ensure that no undesired mutations had resulted during PCR amplification.

Purification of mutant APH(3')-IIIa proteins

Mutant APH(3')-IIIa proteins were purified as previously described [11]. Further purification was required for mutants His82Ala and His188Ala. Pure protein was obtained by application of fractions to a Superdex 200 (HR10/30) column at a flow rate of 0.4 ml min^{-1} in 50 mM Tris HCl pH 8.0, 1 mM EDTA and 200 mM NaCl. Enzyme activity was analyzed by coupling the release of ADP to a pyruvate kinase/lactate dehydrogenase reaction and the initial rates obtained were used to determine the steady state kinetic parameters, as described in McKay *et al.* [11].

Western analysis

Cultures (200 ml) of *E. coli* BL 21 (DE3)/pETSACG1 and *E. coli* BL 21 (DE3)/pAPHH188A were grown from overnight innocula at 37 °C (250 rpm) and induced as previously described [11]. Cells were harvested by centrifugation, lyzed in a French pressure cell and the

lysate fractionated by centrifugation (12 000 x g for 20 min). The insoluble pellet, P1, was resuspended in 5 ml of lysis buffer (50 mM Tris HCl pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 0.1 mM DTT and 0.1 % SDS). The protein concentration of both the supernatant (S1) and P1 fractions were determined and identical amounts of total protein (2 μg) were applied to an SDS-polyacrylamide gel (15 %). Proteins were electroblotted onto nitrocellulose and probed with rabbit anti-APH(3')-IIIa polyclonal antibody (McKay and Wright, unpublished data). Blots were then incubated with an alkaline-phosphatase-conjugated donkey-anti-rabbit (IgG) antibody (Jackson Immuno Research) and developed using 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt/nitroblue tetrazolium chloride (GIBCO/BRL).

Survey of chemical modifiers

The effect on aminoglycoside kinase activity after treatment of wild-type APH(3')-IIIa with N-ethylmaleimide (500 μM), *p*-chloromercuribenzoic acid (500 μM), phenylglyoxal (2.5 mM), 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (2.5 mM), DEPC (10 mM), and tetranitromethane (2.5 mM) was determined as follows. Typically, inactivations were performed in 50 mM HEPES pH 7.5, 10 mM $MgCl_2$ and allowed to proceed for 1 h at 37 °C after which the chemical modifier was removed by passage through a Sephadex G-10 spin column (0.4 ml) and aliquots assayed for enzymatic activity. Treatment of APH(3')-IIIa with phenylglyoxal was performed in 100 mM sodium bicarbonate pH 8.0. The inactivation with tetranitromethane was performed at room temperature.

The rate and stoichiometry of inactivation of APH(3')-IIIa by DEPC were studied in greater detail. Activity remaining was plotted against time and analyzed by nonlinear least squares fitting of equation 1,

$$A = A_{o1}e^{-k_1t} + A_{o2}e^{-k_2t} \quad (1)$$

where A is enzyme activity, A_o is initial activity, k_1 and k_2 are rate constants and t is time. The stoichiometry of inactivation was determined using a final DEPC concentration of 5 mM. Wavelength scans were obtained after a 1 h incubation with DEPC at room temperature and the change in absorbance at 242 nm was determined. The stoichiometry of inactivation was determined using an extinction coefficient of 3200 M^{-1} cm^{-1} [37].

Positional isotope exchange

[γ-^{18}O]-ATP (400 μM) was incubated for 15 min in 50 mM HEPES pH 7.5, 15 mM $MgCl_2$, 13 % D_2O either alone or with APH(3')-IIIa (0.1 nmoles) or with enzyme and 20 μM tobramycin. APH(3')-IIIa activity was quenched by the addition of EDTA to a final concentration of 40 mM and 100 μl of CCl_4. The pH was then adjusted to 9.2. The ^{31}P NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer at 202.456 MHz using a 5 mm broadband inverse probe equipped with a triple axis gradient capability. The spectra were acquired in 36 500 scans over a 7.716 KHz spectral width in 32 K data points (1.062 s acquisition time). A ^{31}P 90° pulse width of 9.0 μs was then used. The relaxation delay was set to 0.5 s. Sample temperature was maintained at 30 °C by a Bruker Eurotherm B-VT 2000 variable temperature apparatus. The free induction decays were processed using exponential multiplication (line broadening: 1.0 Hz) and zero filled to 64 K before Fourier transformation. Chemical shifts are reported relative to external phosphoric acid in D_2O.

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