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Minireview

Phosphoryl transfer by aminoglycoside 3′-phosphotransferases and manifestation of antibiotic resistance

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Abstract

Transfer of the γ-phosphoryl group from ATP to aminoglycoside antibiotics by aminoglycoside 3′-phosphotransferases is one of the most important reactions for manifestation of bacterial resistance to this class of antibiotics. This review article surveys the latest structural and mechanistic findings with these enzymes.

Keywords: Aminoglycoside antibiotics; Antibiotic resistance; Mechanism of phosphate transfer

1. Introduction

In a lucent review article in 1987, Westheimer pointed out that organic phosphates are ubiquitous in biological systems and asked the question of what they do in nature. He provided an answer to this rhetorical question: "almost everything" [1]. Indeed, organic phosphates are widely present among biological molecules and the diversity of their functions is quite remarkable.

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Phosphorylation of small molecules is believed to have come about in part to trap metabolites within the cytoplasm as water-soluble species. Thereafter, enzymes evolved to utilize these molecules for various purposes. Enzymic reactions have evolved to take advantage of organic phosphates in E2, E1, S_N2, S_N1, and S_N2' reactions. Phosphates also have been incorporated into the structures of RNA, DNA, and various nucleotides. They are seen in amphiphilic entities that create biological membranes and they are key in many signaling events in biology. In an indirect way, it is this latter function that is of interest to this review article. As will be discussed in this report, this function has given rise to a strategy that bacteria have exploited to great success in devising resistance mechanisms to aminoglycoside antibiotics.

The primary mechanism of resistance to aminoglycoside antibiotics, well-known molecules such as streptomycin, kanamycin, and gentamicin, among others, is their structure modification by three families of enzymes. These are families of aminoglycoside phosphotransferases, aminoglycoside adenylyltransferases, and aminoglycoside acetyltransferases. The second substrates in all three families are nucleotides, for the first two, ATP, and for the third, acetyl-coenzyme A. A review of the reactions by these enzymes is beyond the scope of this report. However, such reviews have appeared in the literature recently [2–5]. Rather, we have chosen to review here the reaction of the best known family of these enzymes, namely aminoglycoside 3′-phosphotransferases (APH(3′)s).

2. Aminoglycoside antibiotics and mode of action

Before we dwell on the function of these enzymes, let us briefly discuss aminoglycosides in general. The first aminoglycoside, streptomycin, was discovered in 1944 by Waksman from *Streptomyces griseus* [6]. Subsequent efforts led to the discoveries of many more aminoglycosides (for a review see [7]). These antibiotics penetrate the bacterial envelope in an elaborate biphasic process that involves active transport by a mechanism that is not understood, followed by a more rapid penetration.

On entry into the cytoplasm, aminoglycosides bind to the ribosome. Spectinomycin and streptomycin, with substantial structure differences to other classes of aminoglycosides, bind in the minor groove at the end of ribosomal helix 34 (H34). Streptomycin strongly binds to the phosphate backbone of 16S rRNA by salt bridges and hydrogen bonds [8]. In contrast to these antibiotics, other members of aminoglycoside antibiotics bind to the helix 44 near the binding sites for the mRNA and tRNA [9]. An interesting feature of this binding is that it traps RNA residues A1492 and A1493 in an extrahelical conformation [9]. This extrahelical position for these RNA bases is believed to be important for the translation process, namely their electrostatic interactions with the codon–anticodon regions of mRNA and tRNA are believed to play a role in discrimination against the near cognate and non-cognate tRNA molecules [10]. The trapping of aminoglycosides at the A site, fixes the two bases in the extrahelical position, hence the system is primed to accept these non-cognate and near cognate tRNA molecules during translation, hence the fidelity of the process is lowered and defective proteins are produced [11,12]. These

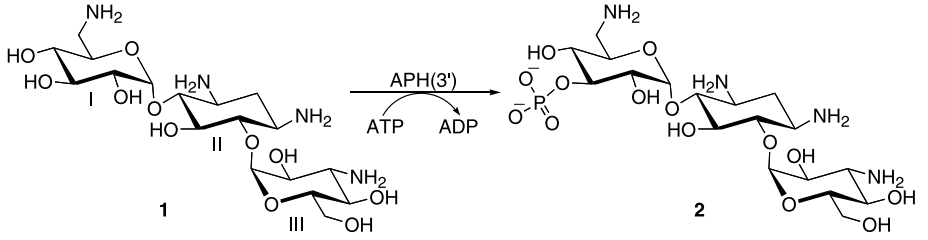
defective proteins are believed to insert into the membrane, whereby the integrity of the membrane is compromised, resulting in damage. It is the damage that causes the more rapid phase of penetration of aminoglycosides into the organism, a set of events that precipitates in the ultimate demise of bacterium [13].

The interactions between the ribosomal RNA and aminoglycosides have been studied by NMR and X-ray structures of the complexes [9,14–18]. These interactions are largely electrostatic in nature involving ion pairing and hydrogen-bonding. The aforementioned enzymic modifications by the antibiotic resistance enzymes manifest their effects in two ways. First, introduction of a large moiety such as the adenylyl group presents steric problems to binding of the modified drug within the limited space in the ribosomal sites. Second, even introduction of relatively smaller functionalities, such as the acetyl and the phosphoryl groups, are detrimental as these disrupt important electrostatic interactions that are necessary for binding. In the case of the phosphoryl group, there should exist an actual repulsion between the negatively charged phosphate monoester on the drug and the phosphate diesters on the backbone of the ribosomal RNA. These contributions to abrogation of binding were studies recently for four of these modifications of the aminoglycoside structure [19]. The modifications were based on the structure of kanamycin A. The modified kanamycin A derivatives were able to bind to the ribosomal A site with dissociation constants that were elevated by 600- to 2000-fold over that of kanamycin A. It is worth noting that among these modified kanamycin A derivatives, the ones with phosphoryl group manifested the largest effect [19].

3. Aminoglycoside 3′-phosphotransferases

The family of aminoglycoside 3′-phosphotransferases is the most widespread among pathogens and is responsible for the demise of kanamycin as a therapeutically important antibiotic. The reaction of this enzyme with kanamycin A (**1**) is depicted in Scheme 1.

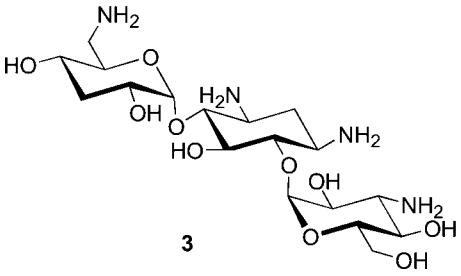
The origin of this group of phosphotransferases is of interest. In groundbreaking work by the Wright and Berghuis groups it was shown that the three-dimensional structure of the type IIIa enzyme from *Enterococcus faecalis* (APH(3′)-IIIa) resembles those of eukaryotic protein kinases such as cyclic-AMP-dependent protein kinase (cAPK) and glycogen phosphorylase kinase γ-subunit [20,21]. Hence, it is



Scheme 1. Aminoglycoside 3′-phosphotransferase-catalyzed reaction.

likely that an existing enzymic reaction that utilized transfer of the γ-phosphoryl of ATP to an amino acid on a protein substrate was subverted for the purpose of the advent of resistance to antibiotics. The duplicated gene for this enzyme would have evolved on an evolutionary tangent that ultimately gave rise to a bona fide determinant for antibiotic resistance. It is of interest to note that Wright has shown that APH(3′)-IIIa is capable of carrying out the transfer of phosphate to protein substrates [22]. Furthermore, some protein kinase inhibitors, such as genestein, quercetin, and isoquinolinesulfonamides, also inhibit APH(3′)-IIIa [23]. Hence, the kinship between the two groups of enzymes has been well established, and the evidence goes beyond the structural similarity of the two groups of enzymes.

The deleterious reaction of this enzyme from a pharmaceutical perspective stimulated research on preparations of aminoglycoside variant that were not affected by it. The semisynthetic 3′-deoxy aminoglycosides, tobramycin (**3**) and dibekacin (**4**), are competitive inhibitors of kanamycin phosphorylation by APH(3′)-IIIa [7,24], as they lack the acceptor for the phosphoryl group.



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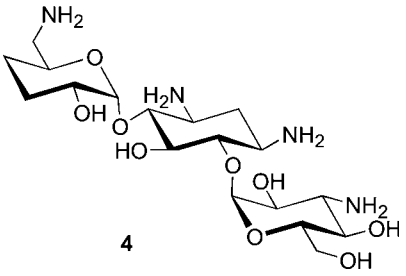

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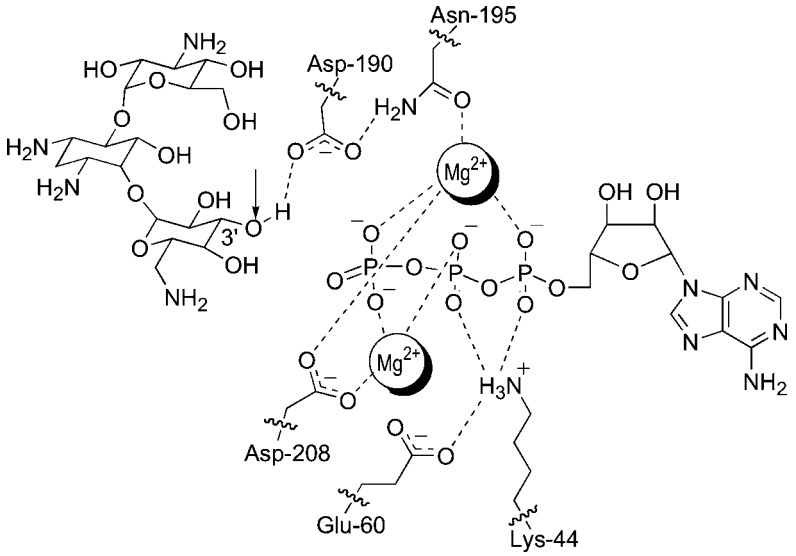
4. Structural information on aminoglycoside 3′-phosphotransferases

APH(3′)-IIIa was the first of these proteins to have been crystallized [20]. APH(3′)-IIIa consists of two domains, a small N-terminal lobe and a large C-terminal lobe, connected by a short hinge segment. The N-terminal lobe is composed of a five-stranded antiparallel β-sheet and two α-helices. The C-terminal lobe is made up of six helices and four short β-strands and provides the aminoglycoside-binding site. The nucleotide-binding site is located in the deep crevasse between two lobes. The nucleotide-binding pocket of APH(3′)-IIIa is aligned with six strictly conserved residues among APH(3′)s: Ser27, Lys44, Glu60, Asp190, Asn195, and Asp208 (Scheme 2). Ser27 in the nucleotide-positioning loop, facilitates bond breakage of the ATP γ-phosphate [25]. Lys44 interacts with the α- and β-phosphate of ATP by electrostatic interactions resulting in enhanced binding affinity of ATP [20]. Glu60 is important for positioning of Lys44 for optimal ATP binding. Asp190 is critical for both aminoglycoside and



Scheme 2. The active site of APH(3′)-IIIa.

metal ion positioning. Asn195 is a ligand for an Mg^{2+} ion and plays a critical role in ADP release during the rate-limiting step. Asp208 coordinates both Mg^{2+} ions and is essential to the formation and stabilization of the transition state [26].

Recently, the X-ray structure of kanamycin/APH-IIa complex has been published [27]. The three-dimensional structure of APH(3′)-IIa is very similar to that of APH(3′)-IIIa despite displaying only 33% amino acid identity. As in the structure of APH(3′)-IIIa, Asp190 in APH(3′)-IIa is hydrogen bonded to the 3′-hydroxyl of kanamycin. Both the I and II rings of kanamycin (Scheme 1) are located in almost identical positions when the two molecules are superimposed on their C-terminal lobes, although there are some differences in detail. On the other hand, ring III has a relatively different orientation in APH(3′)-IIa compared to APH(3′)-IIIa. These differences would give rise to their somewhat different turnover parameters for the same substrate. The modeling of nucleotide binding to APH(3′)-IIa, shows that the only major difference between the nucleotide-binding sites in the two molecules is the orientation of the nucleotide positioning loop. This loop extends away from the nucleotide-binding cleft, while the loop in APH(3′)-IIIa folds down into the empty ATP-binding pocket of the apo-structure. These loops move in response to ATP binding, but the mobility pattern of each loop might be different in the two enzymes.

5. Mechanism of the phosphotransferase reaction

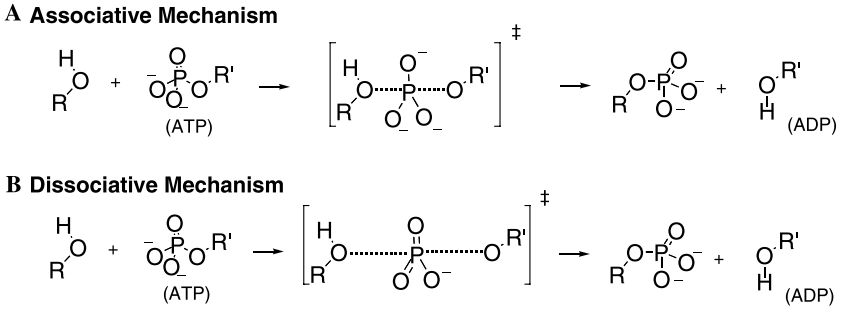
The mechanism of phosphoryl transfer has been described in terms of a continuum between a fully associative (proceeding via a bipyramidal transition

species; S_N2-type) to fully dissociative (involving a metaphosphate-like species; S_N1-type) mechanisms [28–31]. These two possibilities are illustrated in Scheme 3. Two substrates, ATP and the acceptor molecule, align themselves in an enzyme active site. Transfer of phosphate in enzyme-catalyzed reactions is believed to be a *concerted* process, which does not involve an intermediate but has a single transition state. The transition-state species in enzyme-catalyzed reactions have been described by terms such as *tight* (associative) vs *exploded* or *loose* (dissociative) [32].

Many arguments in favor of associative or dissociative transition states for phosphoryl transfer by kinases or phosphatases have been based on the geometry and reaction coordinate distances between the terminal phosphoryl group and its acceptor substrate in ground state enzyme–substrate/inhibitor complexes. The mechanistic details of these enzymic processes are still the subject of considerable debate [26,33–36]. A series of studies have been undertaken to explore the significance of these mechanistic details to the reactions of APH(3′)s.

The best studied APH is APH(3′)-IIIa, found primarily in Gram-positive cocci. The *aph(3′)-IIIa* gene has been cloned from *E. faecalis* [37] and *Staphylococcus aureus* [38] and the protein has been purified from *Escherichia coli* [39]. APH(3′)-IIIa shows a broad spectrum for substrates, including kanamycin, amikacin, neomycin, and butirosin, and confers resistance to a wide range of aminoglycoside antibiotics. Its k_{cat} and k_{cat}/K_m are in the range of 1.0–4.0 s^{-1} and 10^4–10^5 $M^{-1}s^{-1}$, respectively. This enzyme follows a Theorell–Chance mechanism for turnover chemistry, indicative of ordered substrate binding, where ATP binds prior to aminoglycoside and sequential product release of phosphorylated aminoglycoside prior to that of ADP [24,40] takes place. These conclusions were based on results of product inhibition, dead-end inhibition, and solvent isotope and viscosity effect experiments.

Site-directed mutagenesis and affinity labeling studies have been carried out to explore the roles of the conserved residues for APH(3′)-IIIa. As stated earlier, this enzyme is structurally homologous to eukaryotic Ser/Thr and Tyr protein kinases,



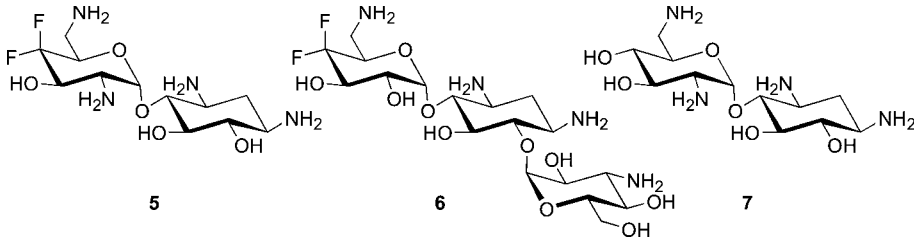
Scheme 3. Transition states of the associative (A) and dissociative (B) mechanisms for the phosphoryl transfer reaction.

despite very low amino acid sequence similarity [20]. It also shares a number of the active site features found in the Ser/Thr and Tyr protein kinases. For example, five amino acid residues (Lys44, Glu60, Asp190, Asn195, and Asp208 in APH(3′)-IIIa) are strictly conserved between APHs and protein kinases [26].

An active site aspartate in protein kinases, corresponding to Asp190 of APH(3′)-IIIa, has been proposed to be the catalytic base for the deprotonation of the substrate hydroxyl. Analyses of the active sites cAPK and Csk have argued that the proposed role is fulfilled by the active site Asp [41,42]. Asp190Ala mutant of APH(3′)-IIIa ($k_{cat} \leqslant 0.0033 \pm 0.0003\ s^{-1}$ for kanamycin A) is over 500-fold less active than the wild-type enzyme ($k_{cat} = 2.04 \pm 0.15\ s^{-1}$ and $k_{cat}/K_m = 1.28 \times 10^5\ M^{-1}s^{-1}$ for kanamycin A) [20]. Asp190Glu mutation, reduces the enzymatic activity to 37- and 1000-fold in k_{cat} ($0.043 \pm 0.001\ s^{-1}$) and k_{cat}/K_m ($1.28 \times 10^5\ M^{-1}s^{-1}$), respectively. Substantially higher catalytic activity of Asp190Glu mutant than that of Asp190Ala mutant indicates that exact positioning of the carboxylate is important for at least 20-fold enhancement of the catalytic rate. Unlike the Asp190Glu mutant, Asp190Asn mutant is isosteric, but without appropriate charge. This mutant variant was more impaired in catalytic activity compared to the Asp190Glu mutant, showing 140-fold decrease in k_{cat} ($0.013 \pm 0.001\ s^{-1}$) compared to the wild-type enzyme [26]. These findings were interpreted to indicate that Asp190 is unlikely to play a role in turnover. The side chain carboxylate of Asp190 may take part in positioning the 3′-hydroxyl group as an acceptor of phosphate, and the initial promotion by deprotonation of the aminoglycoside hydroxyl was deem unnecessary in this case. This notion finds precedent in work on nucleoside diphosphate kinase that suggested that precise positioning of the phosphate acceptor might be a significant contributor to enzymatic catalysis, even when there is little bond formation to the nucleophile in the dissociative transition state [43]. Therefore, the phosphoryl transfer reaction in APH(3′)-IIIa proceeds via a transition state with a dissociative character [25,26].

APH(3′)-Ia is the most common variant in Gram-negative bacteria. APH(3′)-Ia and APH(3′)-IIa have been purified from *E. coli* [44,45]. Steady-state kinetic analysis of APH(3′)-Ia using product-inhibition and alternative substrate kinetics experiments reveals that its catalysis proceeds via an equilibrium-random mechanism. It means that the phosphotransferase activity of APH(3′)-Ia is independent of the order of substrate binding to the active site. In contrast to APH(3′)-IIIa, both APH(3′)-Ia and APH(3′)-IIa also show an ATP hydrolase activity [40,44,45].

The mechanistic possibilities of APH(3′)-Ia and APH(3′)-IIa have been investigated with pre-steady-state kinetic experiments by using two fluorinated aminoglycosides (compounds **5** and **6**) as probes in conjunction with their non-fluorinated parental compounds (**1** and **7**). The presence of the two fluorine atoms at position 4′ of aminoglycosides **5** and **6** would be expected to reduce the nucleophilicity of the 3′-hydroxyl group significantly, impairing the transfer of phosphate, should the nucleophile have a significant participation in the transition species. However, it would influence the reaction to a lesser degree or none in a dissociative reaction.



The fluorinated aminoglycosides were extremely poor substrates for these enzymes, showing approximately 2×10^3- to 1×10^4- and 4×10^3- to 1×10^5-fold reduction of phosphorylation of **5** and **6**, respectively. This is consistent with reduced nucleophilicity of the 3′-hydroxyl by the presence of the fluorine atoms next to the position of phosphorylation. In the case of APH(3′)-Ia, both crystal structures of APH(3′)-IIIa [46] and APH(3′)-IIa [27] indicate that the side chain carboxylate of Asp190, corresponding to Asp198 of APH(3′)-Ia, interacts with the 3′-hydroxyl group by hydrogen-bonding and is likely to activate the hydroxyl for the nucleophilic displacement of the γ-phosphoryl group of ATP. The magnitude of reduction of the catalytic activity of Asp198Ala mutant (k_3(wt)/k_3 (mut) of $(1.5 \pm 0.2) \times 10^3$ for **1** and $(2.2 \pm 0.3) \times 10^4$ for **7**) is within the same range as those for the ratios of the rate constants for the non-fluorinated to fluorinated compounds with the wild-type enzyme. This indicates that activation of the 3′-hydroxyl of aminoglycosides by APH(3′)-Ia is important for turnover chemistry. Investigation with the fluorinated aminoglycosides and the mutant enzyme suggests that, in the case of the two Gram-negative APH(3′)s, the enzymes catalyze their reactions with a significant nucleophilic participation in the transition state.

6. Mechanistic insights from other phosphotransferases

The mechanism of transfer of phosphate has been studied also in protein kinases. The Src kinase (Csk), a non-receptor protein tyrosine kinase [42,47,48], was used in determination of the Brønsted nucleophile coefficient (β_{nuc}), a measure of the role of the nucleophile in the transition state, for the forward reaction and the Brøsted leaving group coefficient (β_{lg}) for the reverse reaction of phosphorylation. Fluorine substitution of the aromatic ring of tyrosine was utilized, because of the relatively small atomic radius and high electronegativity of fluorine that modulates the pK_a of the phenolic hydroxide [34]. A small β_{nuc} (0–0.3) is expected for a dissociative mechanism of non-enzymatic phosphoryl transfer reactions of phosphate monoesters [49], since there is little bond formation between the nucleophile and phosphorus in the transition state, whereas a larger β_{nuc} (0.5–1.0) supports an associative mechanism for non-enzymic phosphoryl transfer of phosphate triesters [50] or methyl transfer reactions [51]. The β_{nuc} values for protein tyrosine kinase Csk were determined by using a series of fluorinated tyrosine analogs as substrates. They were

0.08 and 0.07 for the k_{cat} and k_{cat}/K_m, respectively [47]. These small β_{nuc} values are supportive of a dissociative transition state for this enzymic reaction.

On the other hand, a reaction with β_{lg} value less negative than -0.6 would be attributed to an associative transition state [50]. The β_{lg} value of -0.8 for cleavage of aryl phosphorothioates catalyzed by alkaline phosphatase indicates that the enzyme supports a substantial dissociative transition state [52].

7. Concluding remarks

Phosphate transfer is clearly a key reaction in many biological processes. We have discussed the literature on this reaction as it pertains to aminoglycoside 3′-phosphotransferases and also we have presented evidence on related systems. The essence of the concerted transfer of phosphate from ATP to any acceptor (aminoglycoside antibiotics in the context of this review article) indicates that a continuum exists for the travel of the γ-phosphoryl group of ATP to the acceptor entity. The enzymic reaction proceeds without the involvement of intermediates (local energy minima) and depending on the given case, either scission of the bond between the γ- and β-phosphoryl groups of ATP or the nucleophilic attack by the acceptor on the γ-phosphoryl group of ATP may be facilitated. There would appear to be examples of both in the expansive literature of phosphotransferases.

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