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The Unusual Nucleotide Recognition Properties of the Resistance Enzyme ANT(4′): Inorganic Tri/Polyphosphate as a Substrate for Aminoglycoside Inactivation

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One of the most relevant mechanisms for bacterial resistance to aminoglycoside antibiotics[1] is mediated through their enzymatic modification by acyl- (AACs), phospho- (APHs) or nucleotidyltransferases (ANTs). *Staphylococcus aureus* ANT(4′) is one of the most prevalent enzymes of the last family and promotes the regioselective nucleotidylation of the aminoglucose unit I present in most antibiotics (see Figure 1 a). According to X-ray crystallographic data, the enzyme forms a dimer that has a particular structure in the catalytic region. The ATP molecules (one per monomer) are buried the farthest within this site and the aminoglycosides, exposed to solvent, sitt on the surface of an extended cavity formed by the two subunits of the dimer (Figure 1).[2] The medical relevance of ANT(4′) is highlighted by a recent analysis of clinical isolates insensitive to gentamicin (44 cases) and kanamycin-A (48 cases; Kan-A) that confirmed the presence of this enzyme in 52.3 and 81.3 % of cases, respectively.[3]

A potential strategy to overcome bacterial resistance to aminoglycosides is based upon the coadministration of specific enzymatic inhibitors in tandem with the antibiotic.[4] Although conceptually simple, this approach presents significant difficulties. Aminoglycoside recognition by resistance enzymes relies, to a large extent, upon electrostatic forces, which cause the design of specific inhibitors to be a challenging task. Furthermore, nucleotide binding pockets, like those present in APHs and ANTs, are also present in a significant fraction of human proteins, including key pharmacological targets. In this context, it should be noted that the ANT(4′) nucleotide binding pocket represents an attractive target for inhibitor design, because, in contrast to other resistance enzymes, like the APHs, ANT(4′) lacks significant structural and sequence homology with eukaryotic kinases and phosphatases. This singularity implies that specific ANT(4′) inhibitors should not be cross-reactive with these enzymes and, consequently, might exhibit reduced toxicity.[5]

Although aminoglycoside recognition by resistance enzymes has been the subject of several studies in recent years,[6] the molecular determinants that govern the stability and specificity of nucleotide/enzyme complexes are less well understood. However, this information could provide valuable guidelines for the design of new enzyme inhibitors.[4] Herein, we analyse the molecular determinants for nucleotide recognition by the resistance enzyme ANT(4′). The results obtained reveal that the process involved exhibits distinct and unusual features for ATP-binding proteins which have implications for the design of specific inhibitors.

As a first step in our analysis, the stabilities of the binary (protein/nucleotide) and ternary (protein/nucleotide/antibiotic) complexes were tested. Preliminary studies showed that, in the absence of the antibiotic, the enzyme exhibits residual ATPase activity. To prevent both the enzymatic hydrolysis of ATP (in the protein/ATP binary complex) and the aminoglycoside nucleotidylation (in the ternary complex), we replaced the natural ATP/Mg^{2+} substrate with its nonreactive analogue, ATP/Ca^{2+}. Aminoglycoside binding to ANT(4′) was also analysed for completeness. The K_b, ΔG and ΔH parameters obtained in this analysis are collected in Table 1 and summarised in Figures 2–4.

According to these data, each protein dimer binds two ATP molecules at equivalent sites, with K_b values in the $10^5\,M^{-1}$ range (Table 1, entry 3). The apparent equivalency between the two nucleotide binding sites was expected, since they are located more than 20 Å apart within the ANT(4′) active site. The binding process is enthalpically

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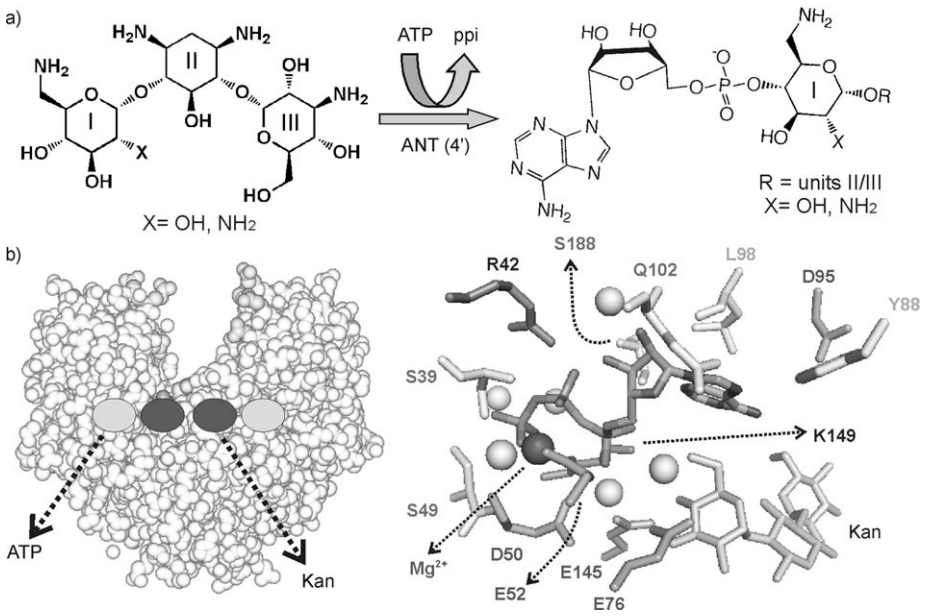

Figure 1. a) Schematic representation of the nucleotidylation reaction promoted by the resistance enzyme ANT(4′). The numbering used for the different aminoglycoside units is shown. In the reaction product, rings II and III are omitted for clarity; ppi = pyrophosphate. b) General organisation of the ANT(4′) active site (left), together with details of the nucleotide recognition site (right). Water molecules are represented as white spheres in the nucleotide recognition site.

Table 1. The thermodynamic parameters measured for the binding of different nucleotide triphosphates and nucleotide fragments to ANT(4′) in the absence (binary complex) and presence (ternary complex) of kanamycin-A, at 25 °C and pH 7.7 in phosphate (20 mM), $CaCl_2$ (2 mM), by using isothermal titration microcalorimetry (ITC). Two equivalent sites per protein dimer were considered. The stoichiometries (n) derived from the ITC curves are also shown.[a]

	Titrating agent		n	K_b [M^{-1}]	ΔG [kcal mol^{-1}]	ΔH [kcal mol^{-1}]
1	Kan-A	−ATP	1.6	1.5×10^4	-5.7 ± 0.2	-3.4 ± 0.6
2	Kan-A	+ATP	2.0	3.0×10^5	-7.4 ± 0.1	-9.5 ± 0.6
3	ATP	−Kan-A	1.8	6.9×10^5	-7.9 ± 0.1	-4.8 ± 0.4
4	ATP	+Kan-A	1.8	6.4×10^6	-9.2 ± 0.1	-12.5 ± 0.7
5	GTP	−Kan-A	1.6	3.4×10^5	-7.5 ± 0.1	-5.0 ± 0.6
6	GTP	+Kan-A	2.0	3.2×10^6	-8.8 ± 0.1	-10.0 ± 0.4
7	UTP	−Kan-A	1.6	1.2×10^5	-6.9 ± 0.1	-2.7 ± 0.5
8	UTP	+Kan-A	1.8	4.5×10^6	-9.0 ± 0.1	-10.6 ± 0.4
9	CTP	−Kan-A	1.5	1.2×10^5	-6.9 ± 0.1	-3.2 ± 0.5
10	CTP	+Kan-A	1.8	3.1×10^6	-8.8 ± 0.2	-10.5 ± 0.5
11	AMPCPP	−Kan-A	1.5	3.1×10^4	-6.1 ± 0.2	-2.8 ± 0.6
12	AMPCPP	+Kan-A	1.6	9.0×10^5	-8.1 ± 0.1	-11.3 ± 0.6
13	ADP	−Kan-A	nd	nd	nd	nd
14	ADP	+Kan-A	nd	nd	nd	nd
15	TP	−Kan-A	1.6	3.0×10^5	-7.4 ± 0.1	-6.5 ± 0.5
16	TP	+Kan-A	1.7	1.9×10^6	-8.5 ± 0.1	-15.4 ± 0.8

[a] AMPCPP = α,β-methyleneadenosine triphosphate; TP = inorganic triphosphate; nd = not determined.

driven, as entropy opposes binding. Interestingly, the protein/nucleotide complex is significantly stabilised by the presence of kanamycin-A (Table 1, entries 3 vs. 4). Moreover, a significant drop in the enthalpy (the process becomes more exothermic) accompanies the observed increase in K_b. Both effects are consistent with the structural information available. Indeed, according to the X-ray crystallography data, kanamycin-A ring I forms a salt bridge with the α-phosphate of ATP and shows clear van der Waals contacts with the nucleotide adenine ring. These additional interactions might provide the energy required to fix the position and conformation of the bound ligands.

As the next step, the nucleotide recognition epitope was identified. For this purpose, ITC experiments were performed employing different nucleotide triphosphates, both in the absence and presence of kanamycin-A. Typical ITC curves are shown in Figure 2 and the derived thermodynamic parameters are collected in Table 1. Several trends are apparent from these data. In the binary complexes, ANT(4′) binds to all of the ligands tested with affinities in the range of $1–7\times10^5\,M^{-1}$ (Table 1, entries 3, 5, 7 and 9), exhibiting a clear preference for those nucleotides containing purine (ATP and GTP) over those that incorporate a pyrimidine (CTP and UTP). Under the experimental conditions employed, the nucleotide base modulates the stability of the complexes, causing differences in ΔG ($\Delta\Delta G$) ≤ 1.0 kcal mol^{-1}. It can be seen that the nucleotide binding strength is, in all cases, significantly increased in the presence of kanamycin-A (ternary complexes, Table 1, entries 4, 6, 8 and 10). Interestingly, for those ligands with a pyrimidine base the stabilisation is more pronounced. Thus, the free-energy difference ($\Delta\Delta G$) between the binary and ternary complexes for both ATP and GTP amount to 1.3 kcal mol^{-1} (Table 1, entries 3 vs. 4 and 5 vs. 6), whereas those for UTP/CTP are 2.1 and 1.9 kcal mol^{-1}, respectively (Table 1, entries 7 vs. 8 and 9 vs. 10). In conclusion, the nucleotide and antibiotic binding sites exhibit more cooperativity if the nucleotide incorporates a pyrimidine base. This effect partially compensates for the intrinsically lower affinity of the protein for the pyrimidine nucleotides, leading to relatively minor stability differences between the different ternary complexes tested (in all cases below 0.4 kcal mol^{-1}).

According to the crystallographic analysis of the ANT(4′)/AMPCPP/Kan-A complex, the triphosphate fragment participates in a significant number of direct and water-mediated polar interactions with the protein′s active site. To fully characterise the nucleotide binding epitope, its influence on the complex stability was tested. Thus, microcalorimetry experiments were performed, employing AMPCPP and ADP, also in the absence and presence of kanamycin-A. Some typical ITC titration curves are represented in the Support-

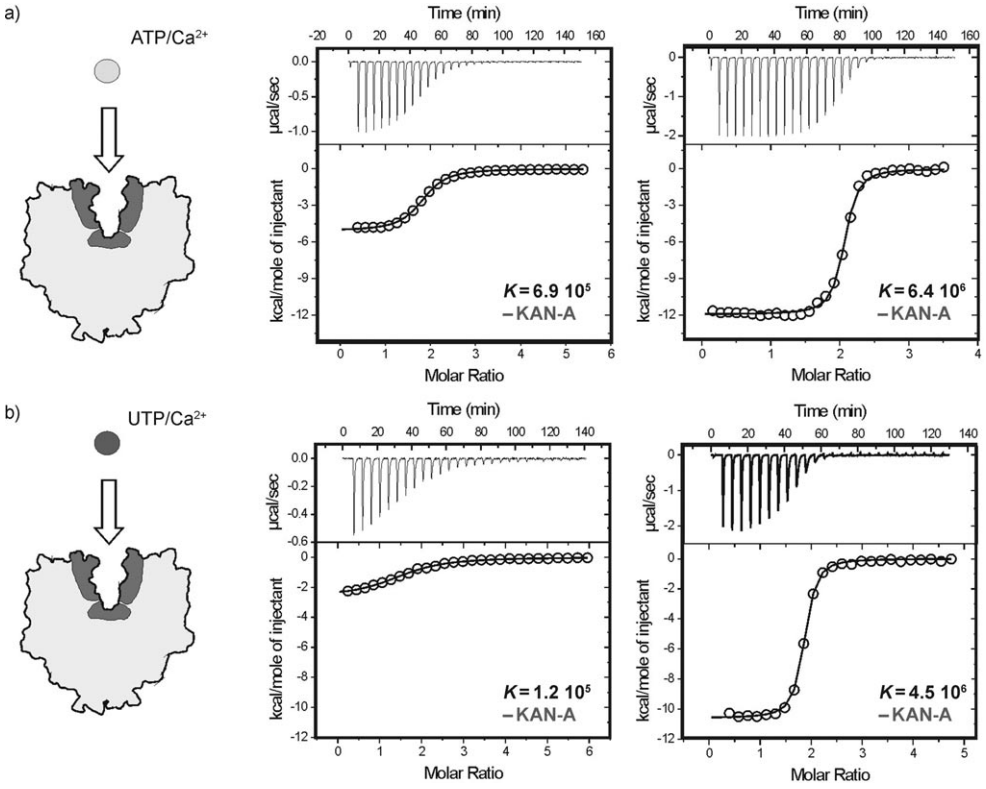

Figure 2. ITC titration experiments performed with a) ATP/Ca^{2+} and b) UTP/Ca^{2+} in the absence (left) and presence (right) of kanamycin-A. Fits were performed by assuming two equivalent binding sites per protein dimer (see Table 1).

ing Information (Figure S1) and the obtained thermodynamic parameters (K_b, ΔG, ΔH and $T\Delta S$) are collected in Table 1 (entries 11–14). The ATP analogue AMPCPP includes a single O/CH_2 substitution in the α phosphate that prevents its cleavage. It should be mentioned that this particular position is not involved in any contact with the protein. Despite this it has a remarkable influence on the binding affinity. Thus, the ITC profiles indicate that the AMPCPP/protein complex is destabilised by 1.8 kcal mol^{-1} with respect to that formed by natural ATP (Table 1, entries 3 vs. 11). This energy penalty is slightly reduced in presence of kanamycin-A ($\Delta\Delta G = 1.1$ kcal mol^{-1}, Table 1, entries 4 vs. 12). A speculative, but plausible, explanation for this is that the geometric and conformational features of AMPCPP prevent an optimal fit of the ligand within the protein binding pocket. In fact, the X-ray data shows that the distance between the reactive OH group of the antibiotic (4-OH of ring I) and the AMPCPP α phosphorus in the ternary complex (5.0 Å) is too large for the reaction to take place. It has been proposed that the enzyme might undergo a conformational change during catalysis to reduce this distance. However, considering the difference in binding affinity between AMPCPP and ATP reported herein, it is also possible that the location of the former within the enzyme active site does not exactly reflect that of natural triphosphate substrates.

For ADP, the ITC data did not reveal any significant binding, even in the presence of kanamycin-A (Table 1, entries 13 and 14). Considering the experimental conditions employed, this observation sets an upper limit of 10^3 M^{-1} for K_b, implying that the removal of a single phosphate destabilises the ternary complex by, at least, 4.5 kcal mol^{-1}.

To further demonstrate the relevance of the triphosphate moiety for ATP recognition, ITC experiments were carried out using inorganic triphosphate (TP, Figure 3), which implies the complete removal of the ribose/base nucleoside fragment. It should be considered that this compound exhibits different acid–base properties to the natural ligand and a stronger anionic character at pH 7.7. Strikingly, it binds to ANT(4′) with affinities comparable to those measured for ATP, GTP, CTP and UTP. Moreover, the favourable influence of kanamycin-A on the stability of the ternary complex ($\Delta\Delta G = 1.1$ kcal mol^{-1}, Table 1, entries 15 vs. 16) is also comparable to that observed for the natural ligands (e.g. $\Delta\Delta G =$ 1.3 kcal mol^{-1} for ATP, Table 1, entries 3 vs. 4). This behaviour reveals that the triphosphate fragment constitutes almost all of the binding epitope of the nucleotide and its integrity appears essential to the preservation of the binding affinity. Indeed, the organic nucleoside fragment only slightly modifies the stability of the complex, causing changes in ΔG of less than 1.0 kcal mol^{-1}. These conclusions are summarised in Figure 4.

The unusual binding properties exhibited by ANT(4′) led us to consider whether inorganic triphosphate could be used as a cosubstrate in the inactivation of natural aminoglycosides. In order to test this hypothesis, the time evolution of a

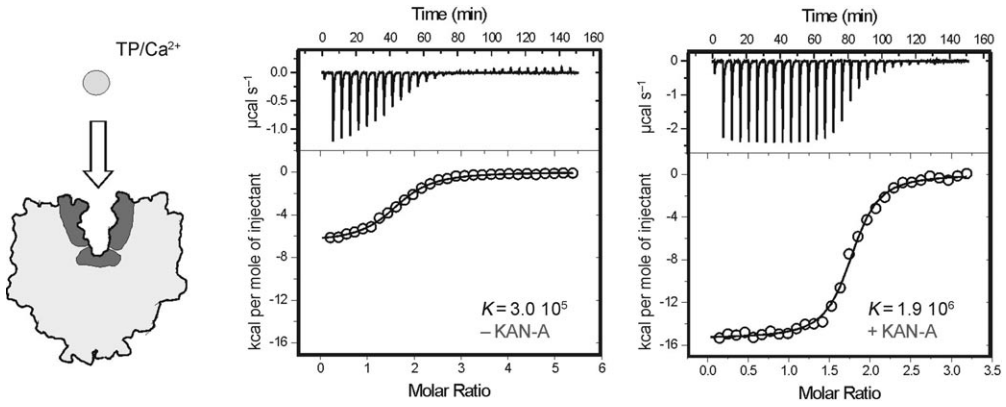

Figure 3. ITC titration experiments performed with inorganic triphosphate/Ca^{2+} in the absence (left) and presence (right) of kanamycin-A. Fits were performed by assuming two equivalent binding sites per protein dimer (see Table 1). TP = inorganic triphosphate.

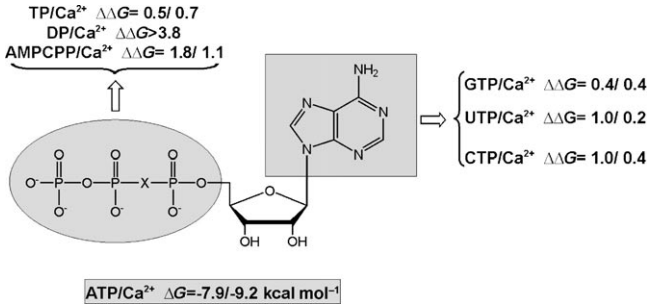

Figure 4. Characterisation of the nucleotide binding epitope by ITC. Differences in ΔG ($\Delta\Delta G$, kcal mol^{-1}) with respect to ATP caused by substitution of the adenine or the triphosphate moieties, in the absence and presence of kanamycin-A (binary/ternary complex), are shown. DP = diphosphate, TP = triphosphate.

kanamycin-B/triphosphate mixture in the presence of $MgCl_2$ (5 mM), after addition of ANT(4′) (10 µM), was monitored by ^{1}H NMR spectroscopy (Figure 5). It was shown that the antibiotic is, indeed, modified to give a single product, which was isolated and characterised by ^{1}H NMR spectroscopy and MALDI-TOF mass spectrometry.

According to this analysis, the enzyme promotes the regioselective phosphorylation of the hydroxyl group at position four of the aminoglycoside unit I. It should be noted that this implies a change in the normal activity of ANT(4′), so that it functions as a phosphotransferase instead of a nucleotidyltransferase. Although this result is somewhat surprising, its biological relevance is unclear, as inorganic triphosphate is not present in living cells. In contrast, longer phosphate oligomers can be found in both prokaryotic and eukaryotic organisms. Although inorganic polyphosphate was first dismissed as a molecular fossil, more recent studies have demonstrated that it still plays important roles; it is a substrate in kinase reactions, provides a reservoir of phosphate, chelates metals and processes/degrades mRNA.[7] Taking this into account, we tested the feasibility of kanamycin inactivation in the presence of longer phosphate oligomers. The results obtained (Figure S2 in the Supporting Information) unambiguously demonstrate that they can also

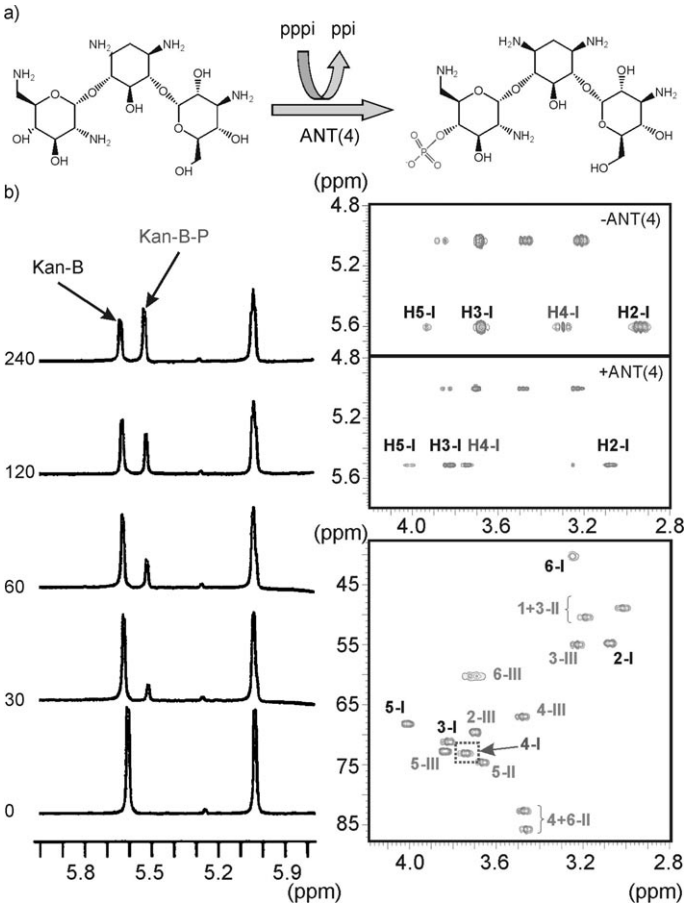

Figure 5. a) Regioselective phosphorylation of kanamycin-B promoted by ANT(4′) in presence of inorganic tri- or polyphosphate. b) left: time evolution of an inorganic triphosphate (2 mM)/kanamycin-B (2 mM) mixture in the presence of ANT(4′) (20 µM) and $MgCl_2$ (5 mM) at 35 °C and pH 8.0 (at lower pH values the triphosphate/kanamycin-B complex precipitates). Right: key region of TOCSY experiments measured for kanamycin-B and the reaction product at pH 4.0. A significant shift downfield of the proton signal at the phosphorylated position (H4-I) is apparent. An HSQC spectrum of the reaction product is shown in the lower right corner.

be employed by the enzyme to promote the regioselective monophosphorylation of the antibiotic. From a molecular

recognition perspective, this observation provides valuable information about the topological features of the ANT(4′) nucleotide-binding region; it must tolerate the extension of the triphosphate fragment present in ATP to facilitate the accommodation of longer oligomeric chains (Figure 6). Interestingly, close inspection of the ANT(4′) active site reveals the presence of a narrow channel adjacent to the nucleotide-binding region. Solvated molecular dynamics simulations performed on the free and polyphosphate-bound enzyme confirmed that its dimensions are sufficient to allow the propagation of the polyphosphate chain out of the enzyme active site. This protein region might also constitute a natural exit for the pyrophosphate that results from the aminoglycoside nucleotidylation reaction.

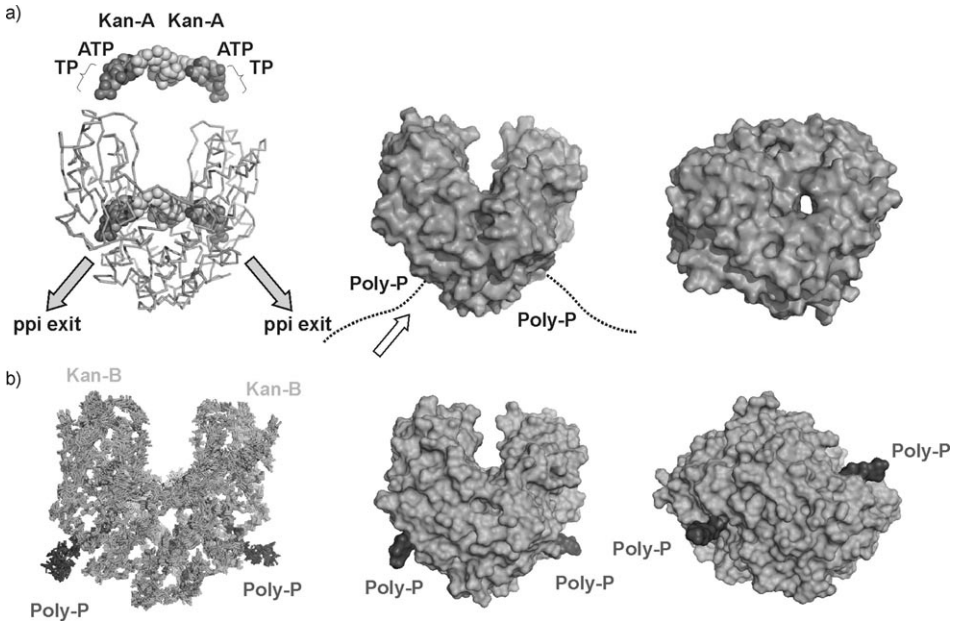

Figure 6. a) Proposed exit site for the pyrophosphate (ppi) fragment in the adenylation reaction. This region might also allow access for the polyphosphate chain to the enzyme catalytic region. The proposed channel is highlighted on the right, in a snapshot taken from a 5 ns solvated molecular dynamics (MD) simulation of the free enzyme. b) Superimposition of 30 structures of the proposed ANT(4′)/kanamycin-B/polyphosphate ternary complex taken from a 5 ns solvated MD simulation. Two different views of a single snap-shot are represented on the right.

We have demonstrated that nucleotide binding to the resistance enzyme ANT(4′) is exclusively mediated by the inorganic triphosphate fragment. Intriguingly, this fragment, along with longer oligomers, can be employed in aminoglycoside inactivation. Both qualities are infrequent for ATP binding proteins (a few examples, such as the myosin ATPase, have been described) and are particularly infrequent in resistance enzymes. The unusual preferences exhibited by ANT(4′) should have ramifications for understanding the evolution, selection and dissemination of the genes in these important enzymes and might suggest a possible evolutionary connection with ancient polyphosphate kinases. Finally, these results have clear implications for the design of specific inhibitors that target the enzyme ATP-binding region. Most known kinase inhibitors bind to the ribose/adenine-binding pocket and therefore, incorporate aromatic or hydrophobic fragments.[8] In ANT(4′) a different strategy should be employed. According to our data, efforts should be exclusively devoted to the mimicry of the inorganic fragment of nucleotides. Indeed, the extended nature of this protein region (i.e., the ability to also bind longer oligomers) suggests that the design of high affinity ligands might be feasible.

As a final point, it should be considered that the design of bioisosteres of the triphosphate side-chain represents a challenging task.[9] Ideally, these analogues should be chemically stable and neutral to facilitate cell permeability. Sugar triazolyl fragments and bicyclic oxazolidine rings have been employed in the past to mimic the Mg^{2+}-triphosphate side-chain complexed to different enzymes.[10] Interestingly, some of the obtained derivatives exhibited moderate inhibitory properties. These results illustrate that the replacement of triphosphate by neutral organic fragments is, indeed, feasible. Current efforts towards this end are currently under way in our group.

Experimental Section

A detailed description of the experimental procedures has been included in the Supporting Information.

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Keywords: aminoglycoside inactivation · antibiotics · enzymes · isothermal titration calorimetry (ITC) · nucleotide binding · nucleotides

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