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Thermodynamic Studies of Aminoglycoside Antibiotic-Enzyme Interactions

[Aminoglikozit – Antibiyotik Enzim Etkileşimlerine Yönelik Termodinamik Çalışmalar]

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ABSTRACT

In this manuscript, we describe thermodynamic properties of complexes formed between aminoglycoside antibiotics and the enzymes that modify these antibiotics and render them useless against infectious bacteria. Studies with three different enzymes that represent three different catalytic modification reactions for these antibiotics are described. These studies revealed certain general properties of these complexes. Formation of the binary enzyme –AG complexes enthalpically favored and entropically disfavored. However, large exothermic enthalpy compensates the unfavorable entropy yielding a favorable free energy (ΔG) of binding in all cases. The presence of co-substrate increases the affinity of AGs to enzymes. A general selectivity pattern for aminoglycosides were also revealed from these studies such that the aminoglycosides with 2'-NH2 and 6'-NH2 bind to enzymes with higher affinity when compared to those with –OH at these positions.

Binding-linked protonation is also observed in the formation of binary enzyme–aminoglycoside and ternary enzyme–co-substrate–AG complexes. Multiple amino groups of aminoglycosides show up-shifted pKas in enzyme–aminoglycoside complexes compared to free aminoglycosides. Determined intrinsic enthalpy (ΔHint) suggested that, at high pH, protonation of amino groups was the major contributor to ΔHint, however, at neutral pH contributions from protonation/deprotonation of other functional groups were also involved.

Key Words: Aminoglycosides, Aminoglycoside-Modifying Enzymes, Thermodynamics, Enzyme–Aminoglycoside Complexes

ÖZET

Bu makalede aminoglikozid antibiyotikleri ve bunları değiştirerek bakteriyel patojenlere karşı etkisiz hale getiren enzimlerin oluşturdukları komplekslerin termodinamik özelliklerini tanımlıyoruz. Üç temel katalitik modifikasyon reaksiyonunu temsil eden üç farklı enzimin kullanıldığı bu çalışmalarda, enzim–aminoglikozid komplekslerinin bir takım genel özellikleri ortaya çıkarıldı. Elde edilen veriler ikili enzim-aminoglikozid kompleks formasyonunun entalpik açıdan avantajlı, entropik açıdan ise dezavantajlı olduğunu göstermektedir . Bununla beraber incelenen tüm durumlarda yüksek egzotermik entalpi negativ entropiye baskın çıkarak sonuçta avantajlı bir bağlanma serbest enerjisi (ΔG) sağlamaktadır. İlaveten, bahsi geçen enzimlerin genel bir seçicilik özelliği olarak, aminoglikozidlerin 2' ve 6' pozisyonlarında –NH2 grubu bulunduranlarına, bu pozisyonlarda –OH grubuna sahip olanlara göre daha yüksek bir afinite ile bağlandığı da gözlemlendi.

İkili enzim-aminoglikozid ve üçlü enzim-kosubstrat-aminoglikozid kompleks oluşumlarında, birden fazla amino grubunun bağlanma sonucu yükselen pKa değerleri protonasyon reaksiyonunun da tesbit edilmesine yol açtı. Çalışılan tüm sistemlerde, elde edilen intrinsik entalpi rakamları, bazik pH değerlerinde başlıca amino gruplarının protonasyonuna, nötral pH değerlerinde ise ek olarak diğer bir takım fonksiyonel grupların protonasyon/deprotonasyonuna işaret etmektedir.

Anahtar Kelimeler: Aminoglikozidler, Aminoglikozid –modifiye eden enzimler, Termodinamik, Enzim-aminoglikozid kompleksleri

INTRODUCTION

Antibiotic resistance has become one of the most challenging problems in the fight against infectious diseases. Today, with one or more types of resistance developed for every antibiotic, there exists a very thin line of defense against many infectious diseases. Due to emergence of multiply resistant pathogens, some useful applications such as the synergistic use of aminoglycosides and β-lactam antibiotics are no longer as effective as before (1, 2). This situation is exacerbated by the fact that most of the antibiotics discovered or developed in the last 30 years represent only variations of existing classes of antibiotics for which resistance is already developed. Thus, very few antibiotics with substantially different structures or target sites have been discovered. Determination of "static" structures of antibiotic–target complexes provides details of structural features of these complexes. However, it is becoming increasingly clear that knowledge of the dynamic and thermodynamic properties of such complexes is essential for a more meaningful drug design campaign to combat antibiotic resistance.

In this review, we will address thermodynamic studies of a very large group of antibiotics, namely aminoglycosides, and their complexes with the equally large group of enzymes that cause resistance to these antibiotics. A brief introduction to aminoglycoside antibiotics and the enzymes that modify these antibiotics is provided in the following sections.

Aminoglycoside Antibiotics

Aminoglycosides (AGs) are quite flexible and charged molecules (Figure 1). They can bind different targets in specific ways (3-6). In bacteria, they bind to the bacterial 30 S ribosomal subunit and interfere with protein biosynthesis, which eventually leads to cell death (7, 8). Aminoglycosides bind to the A site and interact with specific bases of the rRNA (RNA) (9, 10).

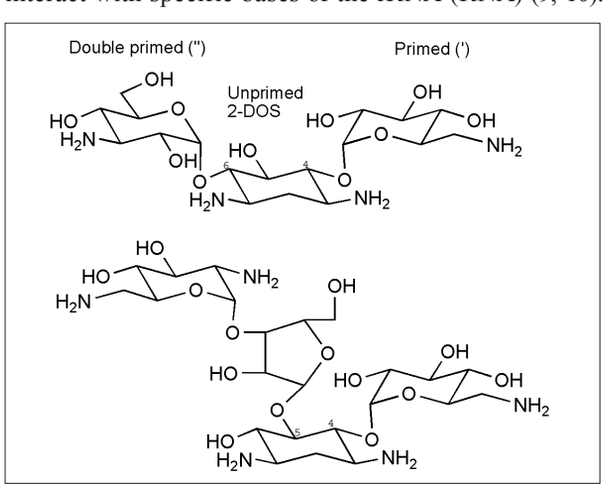

Figure 1. Structures of two aminoglycosides representing aminoglycosides with a 4,6- (kanamycin A-top) and 4,5-substituted (neomycin B-bottom) 2-deoxystreptamine ring. Common nomenclature used for the rings are also shown on top.

The structures of aminoglycosides, bound to RNA or 30 S ribosome, have been solved by NMR and X-ray crystallography (11-15). These studies provide insight into how phosphorylation, nucleotidylation, or acetylation of aminoglycosides may disrupt their interaction with bacterial rRNA. Figure 1 shows representative structures of two large classes of aminoglycosides containing a 4,6-substituted 2-deoxystreptamine (2-DOS) (kanamycin A-top) and a 4,5-substituted 2-DOS (neomycin B-bottom). Commonly used nomenclature for the rings shown at the top. We will refer them as kanamycin and neomycin henceforth.

Aminoglycoside-Modifying Enzymes (AGMEs)

Bacteria produce *O*-phosphotransferases, *O*-nucleotidyltransferases, and *N*-acetyltransferases that modify aminoglycoside antibiotics (16-18). Although there are other modes, enzymatic modification is the major mechanism of resistance to aminoglycoside antibiotics. There are more than fifty AGMEs known today and new enzymes are still being discovered(19). AGMEs are promiscuous enzymes and each can modify a number of aminoglycosides and their semi-synthetic derivatives by acetylation, or nucleotidylation, or phosphorylation (16, 17). Any of these modifications render AGs useless against pathogenic bacteria. Kinetic, biochemical, mechanistic, and crystallographic studies have been performed with several AGMEs and their complexes with AGs (20-29). NMR studies yielded free and enzyme-bound conformations of aminoglycosides bound to four different enzymes (30-34). Conformational aspects of the enzyme-bound aminoglycosides have been reviewed earlier (35). In this article, we will confine ourselves to the description of studies aimed to determine thermodynamic properties of aminoglycoside–enzyme complexes. Although a wealth of kinetic and mechanistic studies are available for many AGMEs, thermodynamic studies of AG binding to AGMEs are very limited. In this article, we will summarize thermodynamic data obtained with three AGMEs: The aminoglycoside-(3′)-phosphotransferase-IIIa (APH) phosphorylates aminoglycoside antibiotics by transferring the terminal phosphoryl group from MgATP to the 3′- and/or 5″–OH of aminoglycosides (21). APH is the most promiscuous aminoglycoside phosphotransferase enzyme and it modifies more than ten different aminoglycoside antibiotics. Aminoglycoside nucleotidyltransferase (2″)-Ia (ANT) is one of the most often detected enzymes in aminoglycoside-resistant bacteria. ANT catalyzes transfer of AMP from MgATP to the 2″-OH of AGs with a 4,6-substituted 2-deoxystreptamine ring (i.e., kanamycins and gentamycins) (20, 36, 37). The third enzyme for which thermodynamic data is available is the aminoglycoside acetyltransferase (6′)-Iy (AAC) [17], which catalyzes the transfer of acetyl group from acetyl-CoA to the 6′-OH of aminoglycosides.

Thermodynamic Parameters of Enzyme–Aminoglycoside Complexes

Aminoglycoside antibiotics are pseudo-saccharides and some of the thermodynamic aspects of their interactions with enzymes are similar to carbohydrate-protein interactions (38, 39). Formation of the binary enzyme–aminoglycoside complex is enthalpically driven and exhibits a disfavored entropic contribution (Table 1). The only exception to this is the binding of two 1N-substituted aminoglycosides (amikacin and netilmicin) to AAC, which proceeds with slightly favorable entropy (40). In all cases, the larger enthalpic contribution dominates and yields a favorable free energy of binding of AGs to AGMEs. The large negative values of TΔS suggest that the sum of total of binding entropy due to solvation effects and rotational, translational, and conformational freedoms of aminoglycosides was greatly reduced in the binary enzyme–aminoglycoside complexes. This is in contrast to AG-RNA interactions, which is accompanied by a favorable entropic contribution (41, 42). A typical data obtained by Isothermal Titration Calorimetry (ITC) for the binding of an AG to an AGME is shown in Figure 2. Binding stoichiometry of 1/1 AG/enzyme is always observed with monomeric ANT and APH (37, 43) and half-stoichiometric binding of AGs to AAC was observed with the dimeric enzyme (40). A similar observation was also made with dimeric APH, which showed that binding of AGs to the second site is much weaker and only detectable with tight-binding AGs (Özen and Serpersu-unpublished data).

Binding studies also revealed that there was no clear distinction between the binding affinity of aminoglycosides with 4,5- substituted or 4,6-substituted 2-deoxysterptamine ring. These studies also showed that neither affinity nor the enthalpy of binding is proportional to the size of AGs. These findings are consistent with NMR studies which showed that the primed and unprimed (2-DOS) rings of enzyme-bound AGs adopt the same conformation regardless of AG and enzyme (34, 35, 44). Thus, these two rings may carry the major contributors to the enzyme-AG recognition/interactions and the rest of the AG molecules contribute variably to the observed thermodynamic properties of these complexes. This also shows that the active site of AGMEs is flexible to accommodate substrates with significant differences in size and/or structure. Another implication of these observations is that dynamic aspects of enzyme-AG complexes may be one of the determinants of substrate affinity to these enzymes.

Another general characteristic property shared by AGMEs is the selectivity toward AGs. The binding of AGs with amino groups at the 2′- and 6′- positions occurs with higher affinity compared to those with –OH at these positions even with enzymes where the chemical modification occurs away from both of these sites. For example, ANT modifies the 2″-OH in the "double primed" ring, which is quite remote from the "primed" ring and yet this enzyme shows strong preference to AGs with amino substitutions at these two sites on the primed ring (Wright and Serpersu-to be published). On the other hand, replacement of –OH at the 3′ site with –H has virtually no effect on the thermodynamic properties of enzyme–AG complexes of all three enzymes (40, 43) (Wright and Serpersu-to be published).

Similarities observed in the thermodynamic properties of enzyme–AG complexes, however, did not extend to their correlation to kinetic parameters of these enzymes; there was no correlation between the observed -ΔH and k_{cat}, K_m, or k_{cat}/K_m for APH (43), while increasing K_b (the association constant) and –ΔH correlated with increase in k_{cat}/K_m and k_{cat} for AAC (ref). In the case of ANT, an increase in K_b was observed with decreasing K_m values and increase in –ΔH with increasing k_{cat} (37).

Table 1. Thermodynamic data obtained with the binary enzyme–AG complexes of AAC, ANT, and APH.

	K_D (μM)	$-\Delta H_{obs}$ [a] (kcal/mol)	$-T\Delta S$ (kcal/mol)	$-\Delta G$ (kcal/mol)
Kanamycin–APH [b]	6.2	33.0	25.6	7.4
Kanamycin–ANT[c]	2.6	15.6	8.1	7.5
Kanamycin–AAC[d]	16	9.6	3.3	6.6
Neomycin–APH[b]	0.26	33.4	24.1	9.3
Neomycin–ANT[c]	0.5	14.3	5.9	8.4
Neomycin–AAC[d]	3.1	14.7	7.2	7.5

a Not corrected for the heat of ionization of buffers

b in Tris-HCl pH 7.5 and at 37°C43.

c in HEPES pH 7.5 at 20°C (Wright and Serpersu-to be published).

d in Tris-HCl pH 7.5 at 27°C40.

The presence of co-substrate increases the affinity of AGs to all enzymes and the free energy of binding (ΔG) becomes more favorable. However, there is a difference in the contribution of ΔH and TΔS to the observed free energy between the enzymes that utilize MgATP or acetyl-CoA as the co-substrate. Studies with ANT and APH showed that, in the presence of metal–ATP, binding of AGs occurs with a reduced enthalpic contribution (37, 43). Entropic contribution, however, becomes more favorable than that of enzyme–AG complexes and over-compensates the reduction in enthalpy yielding a slightly more favorable free energy of binding. Contrary to this, binding of lividomycin to AAC occurs with an increased favorable enthalpy (i.e., larger –ΔH) in the presence of acetyl-CoA while the entropic contribution remains essentially unaltered in AAC (40).

Binding of non-aminoglycoside substrates to the enzymes, studied with Isothermal Titration Calorimetry (ITC), electron paramagnetic resonance (EPR), and fluorescence spectroscopy showed that ATP has very low affinity to APH and ANT in the absence of divalent cation (37) (Wisecarver and Serpersu-unpublished data). Binding studies performed with EPR spectroscopy showed that in the presence of $Mg2^{+}$ or Mn^{2+} the affinity of ATP to both enzymes increases significantly, which is consistent with binding of this substrate to both enzymes as complex with the divalent cation. Although, Mg^{2+} and Mn^{2+} bind to ANT and APH in the absence of ATP, the observed stoichiometry of metal-to-enzyme increases by one in the presence of ATP, again confirming that ATP brings one more divalent cation to the active site. Even though it is determined only with one enzyme, it is also noteworthy that the substrate selectivity of ANT is significantly altered when Mn^{2+} is used instead of Mg^{2+} in kinetic studies (37).

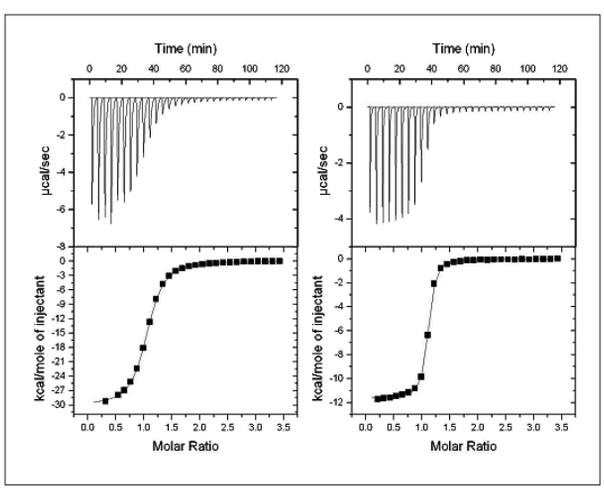

Figure 2. A typical ITC profile for the binding of an AG to an AGME. Top panels show thermograms observed upon titration of ribostamycin to APH in the absence (left) and the presence of CaATP (right). Lower panels show the isotherms for the fitted data. In the presence of CaATP, the increase in the affinity of AG to the enzyme is clearly visible (right panel).

Protonation in Enzyme-Bound Aminoglycosides

Determination of thermodynamic parameters of enzyme–AG complexes is complicated due to the presence of multiple ionizable groups in AGs. Binding of AGs to enzymes yields different ΔH values for the same complex when titrations were performed in buffers with different heats of ionization. This observation indicates that the formation of enzyme-aminoglycoside complexes causes shifts in pK_a of ionizable groups and further protonation/deprotonation occurs in the complex.

In the presence of binding-linked protonation, the observed enthalpy (ΔH_{obs}) includes contribution from various sources according to the equation:

$$\Delta H_{obs} = \Delta H_{int} + \Delta n\,[\alpha\,\Delta H_{ion} + (1-\alpha)\Delta H_{enz}\,] + \Delta H_{bind}$$

in which ΔH_{int} is the intrinsic enthalpy of binding and Δn represents the net proton transfer. ΔH_{obs} denotes the observed binding enthalpy of complex formation in a buffer where ΔH_{ion} describes the heat of ionization of the buffer. The term $\Delta n\,[\alpha\,\Delta H_{ion} + (1-\alpha)\Delta H_{enz}\,]$ represents the heat of ionization of groups from the ionization of buffer and the protein to maintain pH, where α represents fraction of protonation contributed by the buffer (45). In addition, ΔH_{bind} represents the heat of binding of buffer to the enzyme. In the presence of high salt (i.e.,100 mM KCl), ΔH_{bind} is assumed to be zero and the contribution from the ionization of amino acids remains the same at a given pH. Thus, by performing the experiments in buffers with different heats of ionization, one can easily determine ΔH_{int} and Δn. However, note that ΔH_{int} still includes the heat of ionization of groups contributing to Δn (i.e., $\Delta H_{int} = (\Delta H_{int} + \Delta H_{ligand}\Delta n)$ which would represent the true ΔH_{int} only when Δn=0). Studies to determine pH-dependence of the thermodynamic parameters with APH and ANT showed that there was net uptake of protons upon binding of AGs to these enzymes (43) (Wright and Serpersu-to be published). Attempts to fit the data to one or two protonation events failed, suggesting that more than two ionizable groups may have shifted pK_as in enzyme–AG complexes.

In an attempt to understand the type of ionizable groups exhibited shifted pK_as, one may use the determined ΔH at a pH where there is no net protonation occurs (Δn=0) as the true intrinsic enthalpy of the binding. The difference between this value and ΔH observed at a different pH where there is a net proton uptake or release represents the net contribution due the heat of ionization of the groups (ΔH_{group}) with shifted pK_as. Data acquired in studies performed at several different pH yielded enthalpy of ionization values between 6.4 and 10.8 kcal/mol for AG binding to APH and ANT (Wright, Özen, and Serpersu-unpublished data). A common observation

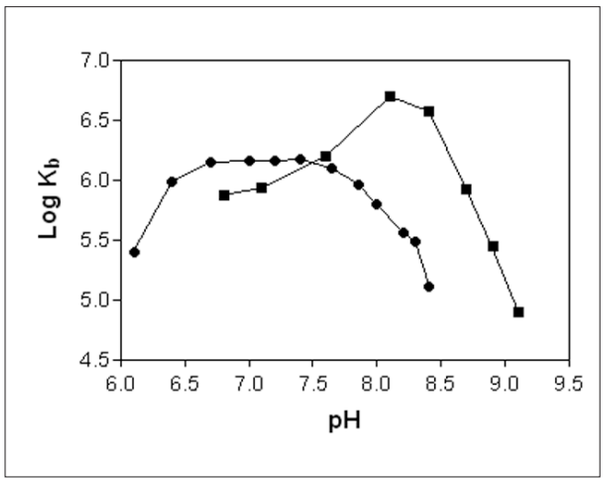

Figure 3. Change in the affinity of neomycin to AGMEs as a function of pH. pH-dependent variation of the association constant (Kb) of the neomycin–ANT (●) and the neomycin–APH (■) complexes.

was made in the studies; ΔH_{group} gradually increased with increasing pH between pH 6.7 and 8.8 reaching the highest value of 10.8 kcal/mol at pH 8.8. 10.8 kcal/mol is consistent with the heat of ionization of amino groups and suggest that the determined ΔH_{group} at this pH represents the proton uptake by amino groups. These are most likely to be the amino functions of AGs. NMR studies of APH–neomycin complex indicate that several amino groups of neomycin show an up shift of ~ 1pK_a unit (Serpersu et al., - to be published). Thus confirming that amino groups of AGs are the main contributors to the determined ΔH_{group} at high pH. However, at neutral pH, protonation/deprotonation of other groups must be contributing to the observed ΔH_{group} yielding an weighted average of ΔH_{ion} of several contributing groups, because the determined ΔH_{group} values are significantly lower than that of an amino group. These observations suggest that at neutral pH, functional groups from the enzymes may also contribute to the observed net protonation and the enthalpy. In fact, determined pK_as of the amino groups in APH-bound neomycin indicate that the observed Δn should have been higher than what is observed experimentally if the only contributor were the amino functions of neomycin. This suggests that some of the proton uptake is compensated by proton release from other groups yielding a lower-than-expected Δn and a lower ΔH_{group}. These observations should serve as a cautionary note that changes in thermodynamic properties are representative of global properties of the complexes and attribution of their differences directly to specific sites/residues may be hazardous. Propagation of binding interactions to remote sites in proteins has been shown before (46).

The protonation state of AGs and enzymes has a significant effect on the binding affinity of AGs to enzymes. The association constant (K_b) of AG–enzyme complexes with ANT and APH shows a strong dependence on pH. Effect of pH on the association constant of enzyme–AG complexes of neomycin with APH and ANT is shown in Figure. Data shown in this figure reveals a complicated pattern; at the low pH regime, there is an increase in the affinity of neomycin to both enzymes, which eventually starts to decrease at high pH. As expected, the latter part of these curves indicates that the decrease in affinity parallels the deprotonation of the amino functions in neomycin. The crystal structure of at least one of these two enzymes (APH) is known, which shows that the active site of APH is rich in negatively-charged groups (aspartic and glutamic side chains) (26). The presence of these residues should facilitate binding of positively charged AGs to this enzyme. The increase in affinity with increasing pH in the low pH regime, however, was unexpected. At low pH, all amino groups of neomycin are almost fully protonated except N3 (pK_a = 5.7 (ref)), which is expected to increase the binding affinity. Charge-charge interactions may not be the dominant effect for the recognition of AGs by AGMEs in low-to-neutral pH range. One may also consider that deprotonation of a positively-charge functional groups in the active site maybe involved at this pH regime. However, examination of the crystal structure of APH-metalATP-neomycin reveals that there are no histidines side chains or any other functional groups with pK_a in or close to this pH range within 9Å of the substrate. These observations clearly show that other interactions and dynamic properties of enzyme–ligand complexes are also important in the recognition of AGs by AGMEs. Studies of several AG-APH complexes by NMR showed that more than 20 backbone amide resonances show different shifts even between the complexes of the enzyme with two AGs that have identical structure with exception of a single site (i.e., -OH vs. -NH_2) (47).

CONCLUSIONS

Data to date, though limited to studies performed with three AGME, demonstrates certain common aspects in thermodynamic properties of enzyme–AG complexes. The generally-shared properties in binding of an AG to AGMEs are:

i) Formation of the binary enzyme –AG complexes enthalpically favored and entropically disfavored. This is contrary to AG-RNA interactions, which occur with favorable entropy.

ii) Large exothermic enthalpy compensates the unfavorable entropy yielding a favorable free energy (ΔG) of binding almost in all cases.

iii) The presence of co-substrate increases the affinity of AGs to enzymes (larger association constants (K_b)).

vi) Aminoglycosides with 2'-NH_2 and 6'-NH_2 bind to AGMEs with higher affinity compared to those with –OH at these positions regardless of the distance

between these sites and the site of modification. On the other hand, replacement of the 3′–OH with –H, which is adjacent to the 2′- position, has virtually no effect on the thermodynamic properties of enzyme–AG complexes.

v) Binding-linked protonation is observed in the formation of binary enzyme–AG and ternary enzyme–co-substrate–AG complexes with APH and ANT. Data suggest that not only multiple amino groups in a given AG display up shifted pK_as but protonation/de-protonation of other functional groups on enzymes also contribute to the observed ΔH_{ion} yielding a different "average" value for this parameter at different pH, which precludes identification of the types of functional groups contributing to the determined ΔH_{ion}.

Although certain global thermodynamic properties of enzyme–AG complexes share general similarities regardless of the enzyme or the aminoglycoside, each AGME has a distinct substrate selectivity pattern. In fact, substrate selectivity observed in enzymes isolated from different sources led to identification of different AGMEs that catalyze the same reaction in earlier years. Global thermodynamic data does not yield direct clues to understand reasons behind the substrate selectivity of each enzyme. Comparative experiments performed with structurally similar AGs can yield valuable results in this respect however. Thermodynamic data also showed that there are distinct differences between some of the thermodynamic properties of enzyme–AG complexes of the same enzyme with structurally very similar aminoglycosides (43). In one case, we were able to use such data to explain substrate selectivity of ANT as described below.

Earlier studies showed that the A and B rings of aminoglycoside antibiotics adopt similar conformations in the active sites of different AGMEs including ANT which led to a hypothesis that these two rings make the most important contacts with enzymes and RNA [35]. Figure 4 shows kanamycin and neomycin superimposed at the primed and unprimed rings as described earlier [34]. The sites of interaction on these rings are indicated by arrows. Thus, even though the site of modification for ANT is on the double primed ring (shown as a ball), interactions of the primed and unprimed rings with the enzyme anchor the antibiotic in the active site. In the case of AGs with a 4,6-disubstituted unprimed ring (kanamycins) this positions the hydroxyl at the 2″ position for nucleophilic attack on the α-phosphate of ATP. For AGs with a 4,5-disubstituted unprimed ring (neomycins), however, neither the 2″- or any other hydroxyl group is close enough for interaction with MgATP (Figure 4). A full rotation of the glycosidic bond between the primed and double primed rings will not bring any hydroxyls of neomycin closer than 3.3 Å to the position occupied by the 2″-OH of kanamycin. Thus, this explains why neomycins can bind to ANT competitively with substrates but are not modified by this enzyme.

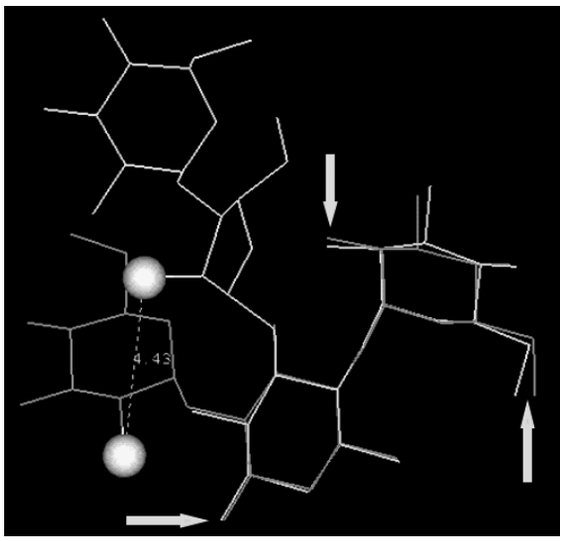

Figure 4. Kanamycin and neomycin superimposed as described earlier (ref) to demonstrate the lack of substrate activity of ANT with neomycins. Anchored points of both aminoglycosides in the active site of ANT are indicated by arrows. This orientation moves the site of modification (2″–OH), represented by balls, on neomycin (cyan) ~4.4 Å away from its position with kanamycin, therby rendering preventing modification of neomycins at this site.

It is clear that thermodynamic, dynamic, and structural studies of enzyme–AG complexes should be combined not only to develop an understanding of global properties of these complexes but also to improve to understand the contributions of specific functional groups to the free energy of binding of different aminoglycoside antibiotics to AGMEs, which will be useful in designing new antimicrobial agents less susceptible to modification and combat more effectively against infectious diseases.

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