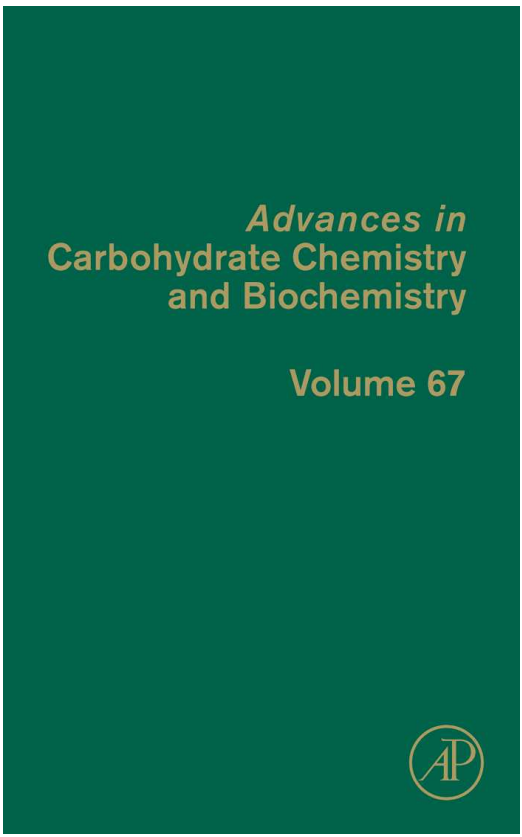

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EFFECT OF PROTEIN DYNAMICS AND SOLVENT IN LIGAND RECOGNITION BY PROMISCUOUS AMINOGLYCOSIDE-MODIFYING ENZYMES

ENGIN H. SERPERSU and ADRIANNE L. NORRIS

Department of Biochemistry, Cellular and Molecular Biology, The University of Tennessee, Knoxville, Tennessee, USA

ABBREVIATIONS

2-DOS, 2-deoxystreptamine; AAC(2′)-Ic, aminoglycoside acetyltransferase(2′)-Ic; AAC(3)-IIIb, aminoglycoside acetyltransferase(3)-IIIb; AAC(6′)-Iy, aminoglycoside acetyltransferase(6′)-Iy; AG, aminoglycoside; AGME, aminoglycoside-modifying enzyme; AMP, adenosine 5′-phosphate; ANT(2″)-Ia, aminoglycoside nucleotidyltransferase (2″)-Ia; APH(3′)-IIIa, aminoglycoside phosphotransferase(3′)-IIIa; CoASH, coenzyme A; GNAT, general control of amino acid synthesis protein 5-related *N*-acetyltransferase; HSQC, heteronuclear single quantum coherence; ITC,

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isothermal titration calorimetry; NMR, nuclear magnetic resonance; RNA, ribonucleic acid; ΔASA, solvent-accessible surface area

I. Introduction

Aminoglycoside antibiotics are a large group of aminocyclitols that are used clinically to treat serious infections. They are among the earliest antibiotics to be used in clinical practice. Streptomycin was the second antibiotic to be discovered after penicillin, and it was used to cure infectious diseases, in particular, tuberculosis.[1] Aminoglycosides bind to the 16S RNA subunit in the bacterial ribosome and interfere with protein synthesis, eventually causing cell death.[2,3] Today, however, their efficacy is threatened, as with all other antibiotics, by an ever-increasing incidence of bacterial strains resistant to their action. The major mode of bacterial resistance to aminoglycoside antibiotics is enzymatic modification of the antibiotic by *N*-acetyl-, *O*-nucleotidyl-, and *O*-phosphotransferases.[4–6] Crystallographic, solution, and computational studies have shown structural features of various RNA–AG complexes that shed light on how modification of these compounds may disrupt their interaction with RNA.[7–13] A number of reviews have been published on various kinetic, biochemical, and structural aspects of the interaction of aminoglycoside antibiotics (AGs) with aminoglycoside-modifying enzymes (AGMEs) and nucleic acids.[4,6,14–20] In this article, we survey dynamic and thermodynamic aspects of AG–enzyme interactions and discuss dynamic properties and unusual effects of solvent in the formation of enzyme–AG complexes and their implications on substrate recognition and promiscuity of these enzymes. Even though some of these enzymes can catalyze side reactions, the term "promiscuity" is used here to define the ability of these enzymes to modify a broad range of AGs.

II. Aminoglycoside Antibiotics

Representative structures of the two major families of aminoglycosides, namely the kanamycins and neomycins, are shown in Fig. 1. The primed ring is 6-amino-6-deoxy-D-glucose in kanamycin A and 2,6-diamino-2,6-dideoxy-D-glucose in neomycin B (henceforth neomycin). The unprimed ring in kanamycin A and neomycin is 2-deoxystreptamine (2-DOS). The double-primed ring is 3-amino-3-deoxy-D-glucose in kanamycin A and D-ribose in neomycin. The fourth ring in neomycin is identified as the triple-primed ring and is 2,6-diamino-2,6-dideoxy-D-glucose. Conformational aspects of enzyme-bound aminoglycosides have been reviewed earlier[21] and will not be repeated here. Suffice it to indicate that the most significant structural feature of

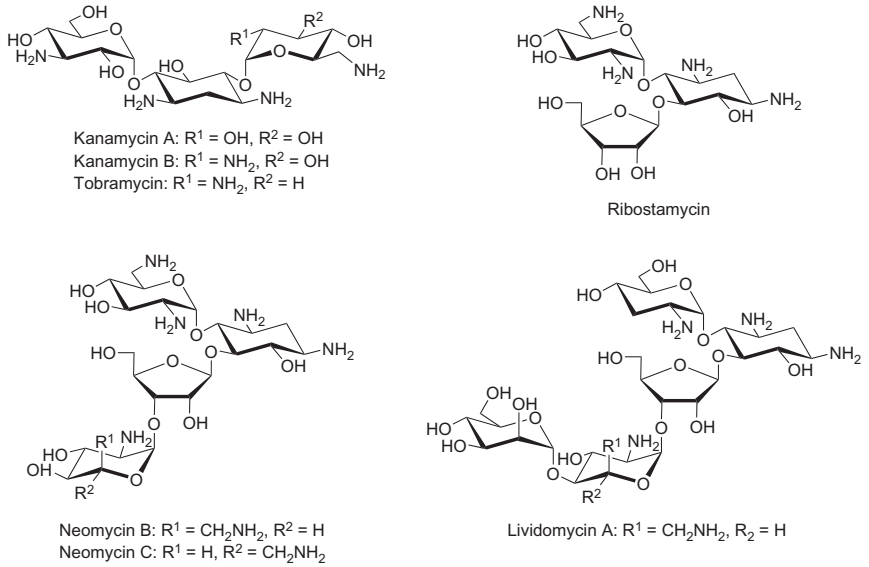

FIG. 1. Representative aminoglycoside structures.

enzyme-bound aminoglycosides is the remarkable similarity in the conformations of the primed and unprimed rings of AGs.

As determined by NMR studies using four different enzymes and a number of structurally diverse AGs, the enzyme-bound conformations of AGs showed that these two rings are superposable, regardless of the enzyme or the antibiotic.[22–25] Structures of AGs, also determined by NMR in complexation with 16S RNA, their natural target[8,11] and by X-ray crystallography in complex with the enzyme APH(3′)-IIIa],[26] also superimpose in the same manner. Figure 2 shows stereo pairs of overlaid conformations of several aminoglycosides bound to four different enzymes (yellow), 16S RNA (blue) determined by NMR and bound to APH(3′)-IIIa (red) taken from the crystal structures of APH(3′)-IIIa. Later, another crystallographic study performed with another aminoglycoside phosphotransferase, APH(3′)-IIa, also showed that the primed and unprimed rings of AGs bound to this enzyme superimpose well with those determined for APH(3′)-IIIa.[27] It is very clear that, while the primed and unprimed rings superimpose remarkably well, the rest of the molecules show a great diversity in their spatial orientation and cover a $\sim 180^{\circ}$ span, even in the active site of the same enzyme.[21] Thus, the primed and unprimed rings of aminoglycosides appear to form the major recognition unit by these enzymes. In fact, this type of analysis allowed us

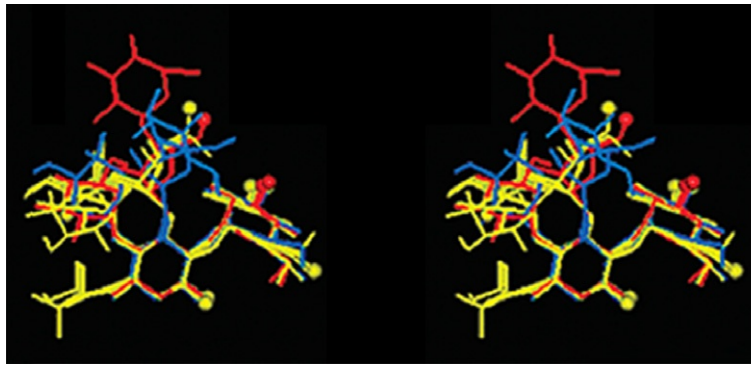

FIG. 2. Stereoview of enzyme/RNA-bound conformations of aminoglycoside antibiotics. In yellow are enzyme-bound AG structures determined with four different enzymes, while blue structures are RNA-bound gentamicin[11] and paromomycin[8] (for simplicity only three rings are shown), all derived via NMR. Red structures are APH(3′)-IIIa-bound kanamycin A and neomycin as determined by X-ray crystallography.[26] All structures are overlaid at the A and B rings. (Reprinted with permission from Ref. 25. Copyright 2002, American Chemical Society.)

to suggest that the inability of the aminoglycoside nucleotidyltransferase (2″)-Ia [ANT(2″)-Ia] to modify neomycins is because the site of modification on neomycins by this enzyme, the 2″-site, moves ∼4.5 Å away from the attacking nucleophile, which would prevent the direct transfer of AMP to AG.[28]

III. AMINOGLYCOSIDE-MODIFYING ENZYMES

Currently, there are more than 50 enzymes known that can modify aminoglycoside antibiotics and render them ineffective against infectious bacteria. They show different levels of substrate promiscuity, ranging from the ability to modify more than a dozen AGs versus just one or two. However, they do show specificity with respect to the site of modification on the AGs. This specificity is incorporated into their nomenclature. For example, aminoglycoside phosphotransferase(3′) denotes an enzyme that phosphorylates the 3-OH group in the primed ring. This in itself, however, still does not specify a unique enzyme until the gene encoding the protein is also included, as in the aminoglycoside phosphotransferase(3′)-IIIa [APH(3′)-IIIa]. This is because multiple enzymes can catalyze the same modification at a given site on AGs. For example, there are more than a dozen enzymes, with varying degrees of homology, that acetylate the N-3 atom of AGs. While some of them are highly promiscuous, such as the aminoglycoside acetyltransferase(3)-IIIb [AAC(3)-IIIb] that acetylates more than 10

structurally different AGs, others such as the aminoglycoside acetyltransferase(3)-Ib can acetylate only gentamicin and fortimycin.[4] Figure 3 shows sequence alignments using a Clustal2.1 algorithm of (a) three proteins that can bind several of the same AGs but catalyze different modifications at different sites and (b) three proteins that catalyze the same reaction at the same site but have little substrate overlap.

Crystal structures of several AGMEs with and/or without bound substrates are also available,[26,27,29–38] many of which have been studied by using kinetic, biochemical, and biophysical techniques.[39–58] However, the molecular basis of differing substrate selectivity by these enzymes remains unexplained. Global parameters determined from thermodynamic or kinetic studies do not address such issues. Static structures provide guidance but fall short in predicting dynamic and thermodynamic aspects of enzyme–ligand complexes with AGMEs. In this article, we address these issues by attempting to describe molecular properties behind the AG selection of these enzymes through a combination of global properties of enzyme–AG complexes with site-specific data.

The superimposability of the primed and 2-DOS rings of all enzyme-bound conformations of AGs described in the previous section, along with the observation that the most buried parts of enzyme-bound AGs are the primed and 2-DOS rings (as demonstrated from the crystal structure of AAC(2′)-Ic[33] with three different AGs), may lead to a conclusion that all or most AGMEs that can modify these antibiotics may have active sites having similar structures and properties. However, in reality, it appears that the dynamic properties of both the ligands and the enzymes play a significant role in the formation of enzyme–AG complexes. These aspects are the main subject of this survey. In the following sections, we discuss the identification of factors that contribute in rendering the formation of enzyme–AG complexes thermodynamically favorable and the roles of these complexes in the process. First, we summarize global thermodynamic properties of various enzyme–AG complexes and then describe identification of the types of molecular interactions that are major contributors to these global properties, along with the sites that are responsible for these interactions.

IV. Thermodynamic Properties of Enzyme–AG Complexes

1. Enthalpy, Entropy, and Gibbs Energy Changes for AG Binding

Thermodynamic data for AG binding to AGMEs are available for four enzymes, the aminoglycoside phosphotransferase(3′)-IIIa [APH(3′)-IIIa],[59–62] the aminoglycoside acetyltransferase(3)-IIIb [AAC(3)-IIIb],[63–65] the aminoglycoside nucleotidyltransferase

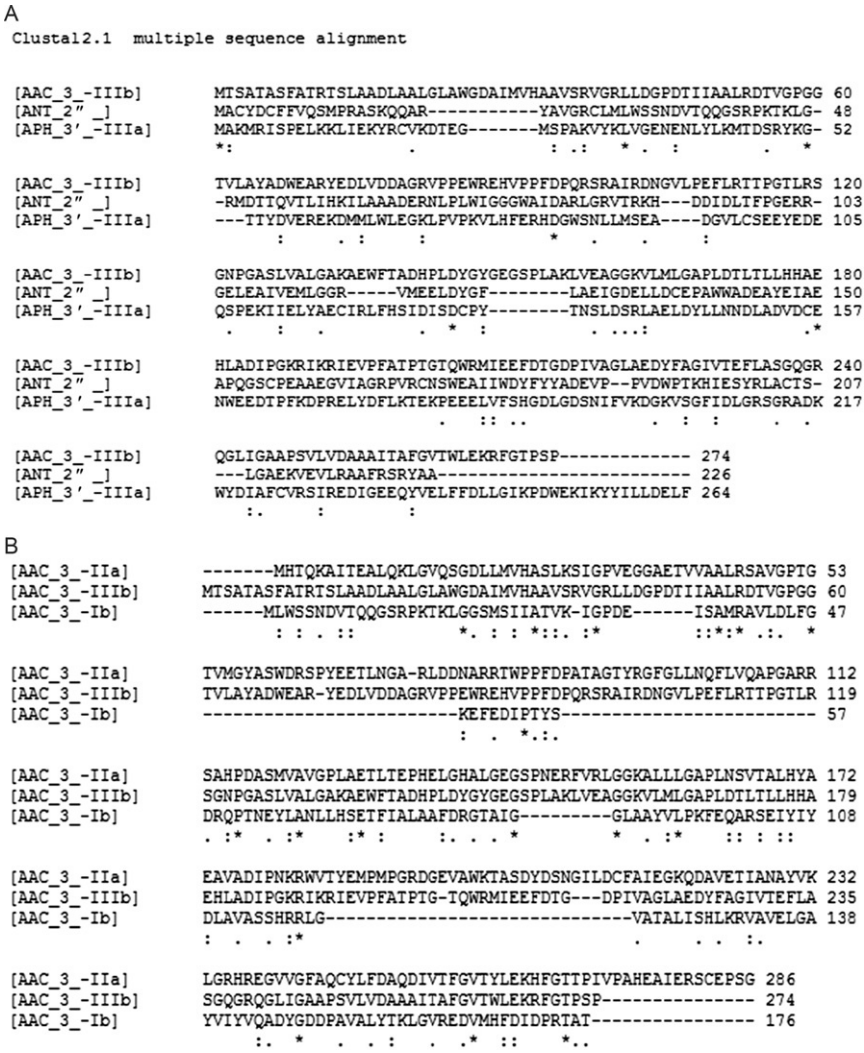

FIG. 3. Sequence alignments of (A) three promiscuous proteins having overlapping AG profiles: APH (3′)-IIIa, AAC(3)-IIIb, and ANT(2″)-Ia; (B) three proteins having high (AAC-IIIb), medium (AAC-IIa), and low (AAC-Ib) substrate promiscuity.

(2″)-Ia [ANT(2″)-Ia],[28,66,67] and the aminoglycoside acetyltransferase(6′)-Iy [AAC (6′)-Iy].[68] These studies showed that binding of AGs to all four enzymes is enthalpically favored and entropically disfavored. The only exceptions are with

acetyltransferases, in which only kanamycin A with AAC(3)-IIIb and amikacin and netilmicin with AAC(6′)-Iy have barely positive $T\Delta S$ values. We should note that data acquired with AAC(6′)-Iy are reported as the observed enthalpy (ΔH_{obs}), whereas the others are reported as the intrinsic enthalpy (ΔH_{int}). As discussed later, ΔH_{obs} includes the contribution from buffer in the form of heat of ionization, and this is strongly dependent on the buffer used.

The binding enthalpy of AGs to AGMEs varies widely in an antibiotic- and enzyme-dependent manner. Differences as large as 40 kcal/mol can be observed for binding of different AGs to the same enzyme [APH(3′)-IIIa].[61] Similarly, binding of the same AG to different enzymes also shows large variations in binding enthalpy. As a consequence of enthalpy–entropy compensation, the entropic contribution ($T\Delta S$) to formation of the complex also shows large, antibiotic-dependent variations. In contrast to these observations, ΔG for binding to all enzymes for all AGs varies within 2–3 kcal/mol over the range between -6 and -8.5 kcal/mol. An example of data from isothermal titration calorimetry is shown in Fig. 4 to demonstrate the differences between a weak and strong binding of AGs to APH(3′)-IIIa.[61]

Binding enthalpies that are significantly different were observed, even for structurally very similar aminoglycosides, and these results allowed identification of sites in AGs that make the most significant contacts with enzymes. For example, the presence of —OH in kanamycin A versus $—NH_2$ in kanamycin B at the 2′-site constitutes the only difference between these two AGs. Their binding enthalpies to all four enzymes show differences ranging from 3.4–3.6 kcal/mol [AAC(6′)-Iy and APH(3′)-IIIa] to 6.9 kcal/mol [AAC(3)-IIIb]. Additional evidence consistent with these data comes from kinetic experiments on the metal-ion dependence of substrate preference with two AGMEs. For APH(3′)-IIIa, AGs with 2′-NH_2 are preferred substrates with MnATP over those with 2′-OH, and this is reversed with MgATP.[69] Exactly, the opposite is true for ANT(2″)-Ia, where the presence of —OH at the 2′-site makes AGs better substrates with MnATP.[66] It is noteworthy that several crystal structures of aminoglycoside-phospho and -acetyltransferases show that 2′-site of AGs does not interact with enzymes.[26,27,33] Therefore, it is difficult to rationalize these data based on static structures of AGMEs. The same conclusion was derived from the crystallographic studies with AAC(2′)-Ic in which the AG-dependent variations in k_{cat} values could not be rationalized from the static structures of the enzyme, as determined with three different AGs.[33] These observations clearly indicate that dynamic features of these enzymes play a significant role in ligand binding and catalysis.

Small differences at other sites in the AG structure also have an impact on the binding enthalpy in an enzyme-dependent manner. To this end, —OH versus $—NH_2$ at the 6′-site (paromomycin and neomycin, respectively) show very large differences

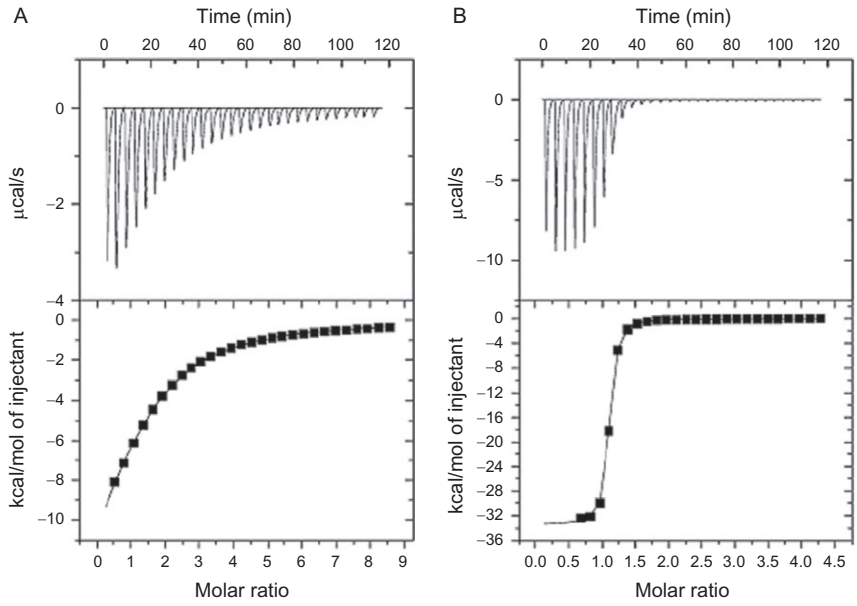

FIG. 4. Typical isotherms of weak (left) and tight (right) binding of AGs to an AGME. The upper panels show the raw data (thermal power). Time integration of the thermal power yields the heat of injection, which is plotted against the molar ratio of ligand to enzyme in the lower panels. Solid lines in the bottom panels constitute the least-squares fitting of the data to a one-site binding model.(Reprinted with permission from Ref. 61. Copyright 2004, American Chemical Society.)

in binding enthalpy ($\Delta\Delta H$) with APH(3′)-IIIa (14.8 kcal/mol) and AAC(6′)-Iy (7.2 kcal/mol), but less than 1 kcal/mol differences with AAC(3)-IIIb (0.3 kcal/mol) and ANT(2″)-Ia (0.9 kcal/mol). Similarly, the 3′-site (where —OH in kanamycin B becomes —H in tobramycin) was most significant for APH(3′)-IIIa (5.7 kcal/mol difference in ΔH_{int} between these two AGs), which is not surprising since this site is the site of modification by this enzyme. However, a 2.1 kcal/mol difference was also detected for AAC(3)-IIIb, which modifies a site on a different ring. On the other hand, AAC(6′)-Iy, an enzyme that modifies a site in the same ring, shows a difference of only 0.9 kcal/mol. As indicated earlier, entropic compensation narrows down the differences to be within 2–3 kcal/mol in the value of ΔG. There was no difference in binding enthalpies of kanamycin B and tobramycin to ANT(2″)-Ia; this enzyme modifies the site most distant from the 3′-OH group. In this case however, the difference in the entropic contribution results in a 1 kcal/mol difference in ΔG for binding of these two AGs to ANT(2″)-Ia.

The binding enthalpy of AGs to AGMEs in the presence of a co-substrate (or co-substrate analogue) shows interesting variations. Counterintuitively, the binding enthalpy of AGs to APH(3′)-IIIa becomes dramatically less favored when CaATP, a competitive inhibitor with respect to the substrate MgATP, is present.[61] The increase in enthalpy (becoming less negative) is between 11 and 20 kcal/mol, and this can be explained only by the surprising observations made in H/D exchange experiments that are described later. ANT(2″)-Ia separates kanamycins from neomycins inasmuch as the binding enthalpy of AGs becomes less favorable for kanamycins and more favorable for neomycins.[28] Interestingly, although neomycins cannot be adenylated by this enzyme, they do bind with high affinity to the protein. In contrast, the binding enthalpy of AGs to AAC(3)-IIIb becomes more favorable for all AGs when the coenzyme A (CoASH) is present.[63] This also holds true for AAC(6′)-Iy, although only one AG, lividomycin, was tested with this enzyme.[68]

2. Proton Linkage

Binding of all AGs to AGMEs is accompanied by a net change in the protonation state of functional groups in the ligand and the enzyme, indicating shifts in pK_as of these groups upon the formation of enzyme–AG complexes.[28,59,61,63] Similarly, shifts in pK_as were also observed in AG–nucleic acid interactions.[70,71] Changes in pK_as cause a net release or uptake of protons, and this triggers a response from the buffer. Therefore, ΔH_{obs} will be dependent on the heat of ionization of the buffer used in binding studies. Determination of intrinsic enthalpy requires the use of several buffers having different heats of ionization and the data to be analyzed by using the equation $\Delta H_{obs} = (\Delta n)(\Delta H_{ion}) + \Delta H_{int}$. This equation is the simplified form of the equation $\Delta H_{obs} = \Delta H_{int} + \Delta n[\alpha \Delta H_{ion} + (1-\alpha)\Delta H_{enz}] + \Delta H_{bind}$.[72,73] The ΔH_{obs} value denotes the observed binding enthalpy of formation of a complex in a given buffer, where ΔH_{ion} describes the heat of ionization of the buffer. The Δn term is the net number of protons transferred as a result of ligand binding, and ΔH_{int} is the intrinsic enthalpy of binding. The term $\Delta n[\alpha \Delta H_{ion} + (1-\alpha)\Delta H_{enz}]$ constitutes the heat of ionization of groups from the ionization of buffer and the protein to maintain pH, where α represents the fraction of protonation contributed by the buffer.[72,73] In addition, ΔH_{bind} constitutes the heat of binding of buffer to the enzyme. In the presence of a high salt concentration (i.e., 100 mM NaCl), ΔH_{bind} is assumed to be zero. Thus, a plot of ΔH_{obs} versus ΔH_{ion} yields a straight line with a slope of Δn and intercept of ΔH_{int}. A representative plot for different complexes of AAC(3)-IIIb is shown in Fig. 5.

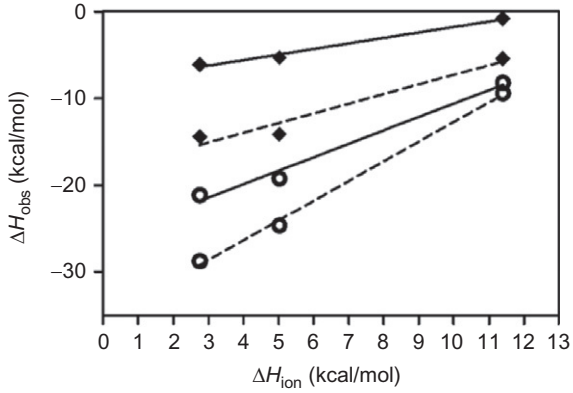

FIG. 5. Dependence of ΔH_{obs} on ΔH_{ion} for the binding of kanamycin B (solid lines) and paromomycin (dashed lines) in the presence (○) and the absence (◆) of CoASH.(Reprinted with permission from Ref. 63. Copyright 2010 American Chemical Society.)

Data available for APH(3′)-IIIa, AAC(3)-IIIb, and ANT(2″)-Ia show that there is no unique pattern for a particular enzyme or AG. The Δn term is different for each AG with a given enzyme, and binding of any AG to different enzymes also yields different Δn values, and these are further altered in the presence of the co-substrate. In general, there is always a net proton uptake when AGs bind to AGMEs. There are only a few exceptions, such as the binding of ribostamycin and amikacin to APH(3′)-IIIa, which occurs with a slight net release of protons, but is reversed in the presence of the metal–ATP co-substrate.[61] In the case of AAC(3)-IIIb, the only exception is formation of the AAC(3)-IIIb–kanamycin A complex, which yields $\Delta n \approx 0$. Formation of the ternary AAC(3)-IIIb–CoASH–kanamycin A complex, however, proceeds with a $\Delta n = 1.3$, denoting a net uptake of protons.[63]

We emphasize that caution should be exercised in the interpretation of the observed Δn values. The ΔH_{int} value determined in this manner still includes the heat of ionization of groups from both the protein and the ligand that are contributing to Δn, and this may represent the true ΔH_{int} value only when $\Delta n = 0$. Even if this condition is met, it still cannot be used as evidence against pK_a shifts in different functional groups because Δn represents the net proton balance. An example of this situation was observed in the binding of neomycin to APH(3′)-IIIa. At pH 6.7, Δn was determined to be ~ 0. However, pK_as of the amine groups of neomycin, as determined by NMR, indicated that the net proton uptake for the ligand alone should have been ~ 0.7 under these conditions. This observation clearly indicates that a net release of protons from the functional groups of the enzyme coincidentally matched the net

uptake of the ligand, yielding a net value of zero for Δn.[59] In fact, computational studies have shown that a large number of ionizable groups alter their pK_as significantly when AGs bind to APH(3′)-IIIa in which ~50% of the residues have ionizable groups (Serpersu and Ullmann, unpublished data). As already mentioned, the binding of kanamycin A to AAC(3)-IIIb proceeds with apparent $\Delta n \approx 0$. However, it is difficult to imagine that no changes will occur in pK_as of any one of the four amine groups of kanamycin A, as well as other functional groups of the enzyme, upon binding. We consider that this represents another coincidental cancellation of protonation and deprotonations under the experimental conditions, as observed with the binding of neomycin to APH(3′)-IIIa. Therefore, attempts to dissect Δn, based on ITC data alone, are likely to be misleading.

3. Heat-Capacity Changes

The most detailed thermodynamic data are available only for two AGMEs, APH (3′)-IIIa and AAC(3)-IIIb. The rest of this article is devoted mostly to the description and discussion of unusual thermodynamic properties of these two enzymes, both of which display very high substrate promiscuity and can modify more than ten structurally diverse AGs. Their dynamic and thermodynamic properties may hold keys to their ability to modify a broad range of AGs. The results may lead to an understanding of the molecular principles underlying the substrate promiscuity of these two enzymes that catalyze different reactions to modify different rings on AGs and show a large overlap in their substrate spectrum, despite the very small sequence homology (<5%) in their primary structure (Fig. 3).

The change in the heat capacity of proteins attributable to a ligand-binding event is affected by several factors, such as hydrophobic interactions, electrostatic charges, hydrogen bonds, intramolecular vibrations, and conformational entropy.[74] Changes in interactions of hydrophobic groups with solvent and alterations of low-frequency vibrational modes of the protein (i.e., stiffening or loosening of the protein) are among the most significant of these effects. Determination of the enthalpy of ligand binding as a function of temperature for a narrow temperature range usually yields a straight line. The change in heat capacity (ΔC_p) can be obtained from the slope of this line.

Although such data are available only for the binding of AGs to APH(3′)-IIIa[60] and AAC(3)-IIIb,[63] extremely surprising results highlighted some of the key thermodynamic factors that govern the substrate recognition and discrimination by these two highly promiscuous enzymes. These observations may reflect some of the general molecular properties of ligand recognition, not only by promiscuous AGMEs but also in the broader context of ligand recognition in general.

There are significant differences in the binding of kanamycin A and neomycin to APH(3′)-IIIa. The ΔC_p is temperature independent for the binding of neomycin to APH(3′)-IIIa, whereas strong dependence is observed for the binding of kanamycin A (Fig. 6). These observations were the first to demonstrate a difference in the interaction of this enzyme between kanamycins and neomycins, as previous kinetic and binding studies did not reveal any parameter that would distinguish interactions of these two classes of AGs with APH(3′)-IIIa. The dependence of enthalpy on temperature is unusually strong for both AGs and yields ΔC_p values of -1.6 kcal/mol/deg for neomycin and ~ -0.7 kcal/mol/deg below 30 °C and ~ -3.8 kcal/mol/deg above this temperature for kanamycin A.[60] These values are much higher than those determined for common carbohydrate–protein interactions, which are usually within the range of -0.1 to -0.5 kcal/mol/deg.[75,76] Neither the unusually negative values of ΔC_p nor the large difference in its values between the binding of kanamycin A and neomycin to APH(3′)-IIIa can be explained by changes in the solvent-accessible surface area (ΔASA) alone. Large conformational changes and/or significantly altered dynamic properties of the protein, such as changes in low-frequency vibrational modes, may have significant impact in the formation of these complexes.

A crystal structure of the enzyme in a binary complex with any AG is not available to help explain these differences. However, the crystal structures of the apoenzyme, nucleotide–enzyme, and enzyme–nucleotide–AG complexes show that no significant domain movements occur upon formation of these complexes.[26] Figure 7 shows superimposed structures of the apo-APH(3′)-IIIa,[31] APH(3′)-IIIa–MgAMPPNP,[36]

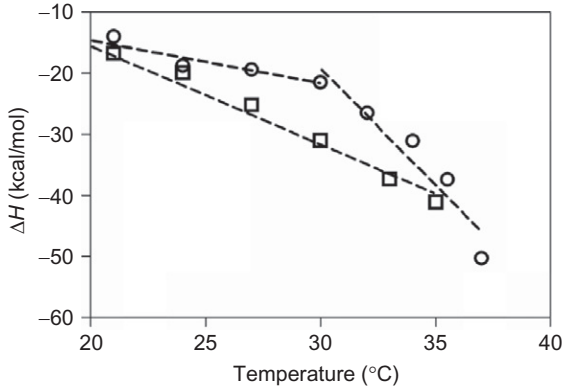

FIG. 6. Change in heat capacity upon interaction of APH(3′)-IIIa with neomycin (□) and kanamycin A (○).Reprinted with permission from Ref. 61. Copyright 2004, American Chemical Society.

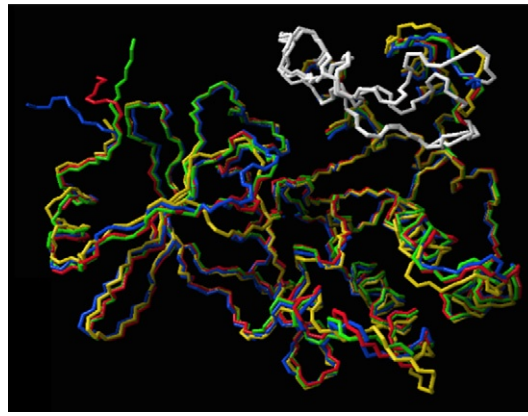

FIG. 7. Superimposed structures of APH(3′)-IIIa in apo (yellow), nucleotide (blue), and nucleotide–AG complexes with neomycin (green) and kanamycin (red).

APH(3′)-IIIa–MgADP–kanamycin A,[26] and APH(3′)-IIIa–MgADP–neomycin[26] complexes. The major region showing a significant variation is the loop formed by residues 147–170 just above the AG-binding site, which is highlighted in white in all of the structures. As indicated by the authors, the caveat is that APH(3′)-IIIa is a ∼30 kDa monomeric enzyme having a strong tendency to form dimers via two intermolecular disulfide bridges. One of these is Cys-156, located in the middle of the loop covering the AG-binding site. In crystal structures of most complexes of this enzyme, the dimer has Cys-156 forming a disulfide bond with Cys-19 of the other monomer in the same unit cell. In other structures, the disulfide bond is with a monomer in the adjacent unit cell. It is not known to what extent these variations may affect the observed differences in the orientation of this loop. In solution, however, formation of the dimer is observed to have a profound effect on the binding affinity of AGs to this enzyme, despite a separation distance of ∼20 Å, as determined from crystallographic studies, between the two active sites. The binding affinity of neomycin to each monomer of the dimer differs by approximately three orders of magnitude.[59] In any case, the lack of domain movements observed in the static structures of the enzyme suggests that changes in dynamics of the protein must be one of the major contributors to ΔC_p, and this conclusion is also supported by NMR data, as described later in Section V.

Aminoglycoside-dependent differences in ΔC_p are also observed for AAC(3)-IIIb in an even more dramatic manner than that observed with APH(3′)-IIIa. For this enzyme, the binding of neomycin causes a decrease in heat capacity, while binding of

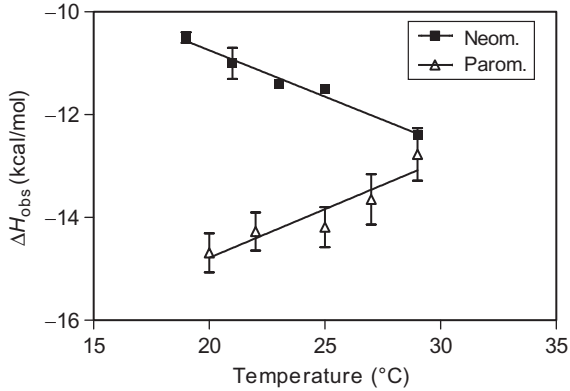

FIG. 8. Changes in heat capacity in the association of AAC(3)-IIIb to neomycin (■) and paromomycin (▲).(Adapted with permission from Ref. 64. Copyright 2011 American Chemical Society.)

paromomycin, a structurally similar AG, causes an increase in the heat capacity[64] (Fig. 8). As discussed later in Section V, these opposite trends are attributed to different conformations of a large loop that is at the AG-binding site of the enzyme. There is only one other case where binding of ligands to a protein causes the heat capacity to change in opposite directions.[77] In that instance, however, the ligands are structurally very different.

4. Solvent Effects

Solvent effects are one of the major contributors to ΔC_p, and the surprisingly large and unexpected AG-dependent differences observed in this parameter suggest that solvent effects may be significant for AG–enzyme interactions. Osmotic-stress experiments, performed by isothermal titration calorimetry (ITC), showed that there is a significant difference in solvent displacement between the binding of kanamycin A and neomycin to ANT(2″)-Ia. While the binding of kanamycin A expelled ~20 water molecules, there was no displacement with neomycin.[28] The crystal structure of AAC(2′)-Ic showed that the binding of tobramycin displaces six to eight water molecules.[33]

The binding enthalpy of kanamycins and neomycins to APH(3′)-IIIa showed a very unusual pattern that had never been observed before. In this situation, the $\Delta\Delta H$ (ΔH_{H_2O}–ΔH_{D_2O}) values between kanamycins and neomycins have opposite signs, such that kanamycins bind with a more favorable enthalpy in H_2O than in D_2O, whereas neomycins bind with more favorable enthalpy in D_2O than in H_2O (Fig. 9).[60]

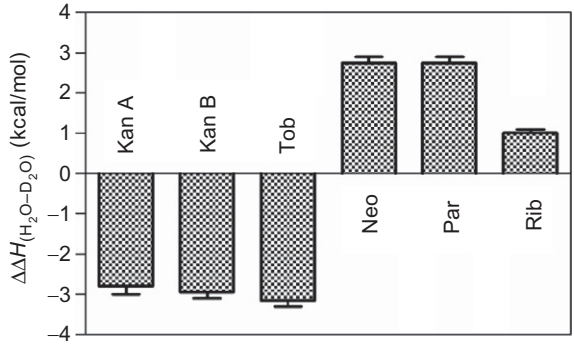

FIG. 9. Solvent-dependent differences in binding enthalpies of kanamycins and neomycins to APH(3′)-IIIa.(Reprinted with permission from Ref. 60. Copyright 2008 American Chemical Society.)

These data suggest that APH(3′)-IIIa uses solvent rearrangement to differentiate between the neomycin-class aminoglycosides and the kanamycin class. This factor may be one of the major contributors to the large differences observed in ΔC_p. It may be recalled that the 2-deoxystreptamine and primed rings of all enzyme-bound aminoglycosides are well superimposed, but differences arise in the rest of the antibiotic structure. Elastic network analysis demonstrated that there are two stretches of backbone residues located in two different domains of APH(3′)-IIIa that show differences in their correlated motions with kanamycin A and neomycin B (yellow segments in Fig. 10).[78]

The nonsuperimposable rings of neomycin and kanamycin A are oriented differently with respect to these two segments, suggesting that this interface is a potential region of solvent rearrangement that is responsible for the class-specific differences observed between kanamycins and neomycins in both $\Delta\Delta H$ and ΔC_p. This segment is highlighted in Fig. 10, which shows the superimposed crystal structures of APH(3′)-IIIa–MgADP–kanamycin A and APH(3′)-IIIa–MgADP–neomycin complexes. Again, these are suggestive of a significant role of protein dynamics and solvent interactions in ligand recognition by this enzyme. This is because, as shown in Fig. 10, both kanamycin A- and neomycin-bound structures of the enzyme, as determined by X-ray crystallography, are superimposable and do not show large differences in solvent-exposed surfaces between the two complexes that would yield clues for explaining such a dramatic solvent effect.

For the formation of enzyme–AG complexes of AAC(3)-IIIb, the solvent effect was again significant, and in some sense even more surprising than that observed with APH(3′)-IIIa. In this instance, a dramatic difference in $\Delta\Delta H$ values was observed for

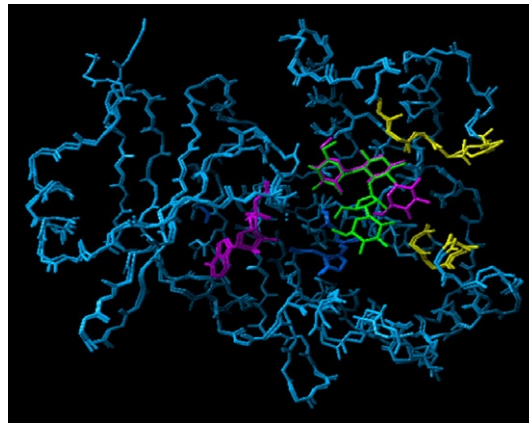

FIG. 10. Superimposed structures of APH(3′)-IIIa–MgADP–neomycin and APH(3′)-IIIa–MgADP–kanamycin A. Neomycin (green) and kanamycin (purple) are on the right side. The purple molecule on the left is MgADP. Yellow highlights those residues showing AG-dependent differences in their correlated motions.

the binding of neomycin and paromomycin to AAC(3)-IIIb in H_2O and D_2O. These two AGs are structurally almost identical, having just a single change at the 6′-site (which is —OH in paromomycin and —NH_2 in neomycin), and yet the binding of neomycin to AAC(3)-IIIb is enthalpically more favored in D_2O than in H_2O, while the binding is exactly opposite for paromomycin.[64] This behavior is, however, only true for temperatures below 27 °C, and this is another very surprising and a first-time observation. These data show that the major contributors to the ΔC_p of the interaction of AAC(3)-IIIb with ligands are temperature and antibiotic dependent. When neomycin binds to AAC(3)-IIIb, the $\Delta\Delta H(\Delta H_{H_2O}–\Delta H_{D_2O})$ shows a strong dependence on temperature such that, at low temperatures, changes in low-frequency vibrational modes on the protein dominate the ΔC_p, but as the temperature increases, the balance between vibrational and solvent contributions shifts increasingly more toward the latter (Fig. 11). However, with paromomycin, there is no change in the $\Delta\Delta H$ value with temperature. This suggests that either solvent effects dominate at all temperatures, or effects from multiple contributions oppose each other and coincidentally cancel out, leaving $\Delta\Delta H$ unchanged. In either case, it is clear that AAC(3)-IIIb responds very differently to neomycin and paromomycin, and solvent reorganization plays a significant role in the AG-recognition process. The unusual interplay of contributions of changes in low-frequency vibrational modes of the protein (namely, stiffening or loosening of the enzyme), and solvent effects and their temperature and

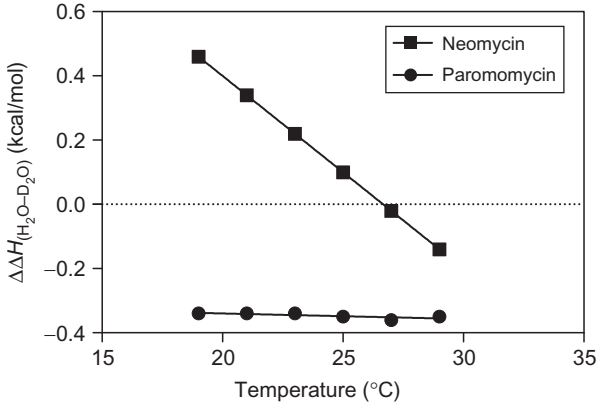

FIG. 11. Temperature dependency of $\Delta\Delta H$ for association of neomycin (■) and paromomycin (●) with AAC(3)-IIIb.(Adapted with permission from Ref. 64. Copyright 2011 American Chemical Society.)

AG dependence on the formation of enzyme–AG complexes, may not be specific to AAC(3)-IIIb because, to the best of our knowledge, there are no such data available for any other enzyme. These observations clearly indicate that dynamic features of the AGMEs play a very important role in rendering the binding of AGs to the enzyme thermodynamically favorable under different conditions. Observations such as these also show that solvent not only plays a significant role in substrate recognition but also has differential effects with each AG. Finally, it is very unlikely that the unusual solvent effects observed with different AGMEs that catalyze different reactions are coincidental. These properties are likely to apply to other AGMEs, or even other promiscuous enzymes in general.

V. PROTEIN DYNAMICS IN SUBSTRATE RECOGNITION AND SUBSTRATE PROMISCUITY OF AGMES

From fluid-breathing motions to conformational changes, structural dynamics are a property of proteins that is often important, if not critical, to their function. As many AGMEs bind to and/or modify a large number of structurally diverse AGs, it follows that dynamics could play a role in their promiscuous nature. For APH(3′)-IIIa, crystal structures of the apoenzyme as compared with the nucleotide or nucleotide/antibiotic complexes reveal that the protein is well structured in all features except that a 23-residue loop is altered in response to binding to the antibiotic (Fig. 7). Thus it was

initially proposed that this enzyme's promiscuity results from an open antibiotic-binding site. Nuclear magnetic resonance experiments with uniformly ^{15}N-labeled APH(3′)-IIIa demonstrate that the apoenzyme is undergoing interconversion between several conformations, as the ^{15}N–^{1}H HSQC spectrum shows all the hallmarks of an unstructured or molten-globule form of a protein. Addition of an antibiotic causes significant changes in the spectrum, indicating that formation of the binary enzyme–AG complex induces a well-defined structure in the enzyme. The nucleotide cannot accomplish this change. In fact, the addition of metal–nucleotide to the apoenzyme or enzyme–AG complexes yields NMR spectra that are consistent with increased flexibility and solvent exposure in the protein.[79] These data in themselves strongly suggest that the protein is very flexible and thus can accommodate a large repertoire of ligands via structural dynamics. A similar observation has been made with the aminoglycoside acetyltransferase(6′)-Ii, which also showed a highly overlapping NMR spectrum that showed much higher resolution after the binding of CoASH.[80] These results are also consistent with this enzyme being highly dynamic in solution.

The dynamic properties of APH(3′)-IIIa were probed by H/D exchange. In the apoenzyme, $>90\%$ of all backbone amide groups, even those known to be involved in hydrogen bonds of the secondary structure, can exchange their proton for deuterium within 5 min of exposure to D_2O, while the remainder undergo complete exchange within 10 h.[79] MgAMPPCP alone did not provide much protection either; however, binding of antibiotics allows 30–40% of residues to be protected from solvent for >90 h, where both the sites and degree of protection is antibiotic dependent (Fig. 12).

Moreover, protection patterns and antibiotic-dependent chemical shifts are not confined to the antibiotic-binding region but instead reach to diverse regions of the protein. The chemical properties and the location of these residues in this enzyme show a very interesting behavior. In this context, $\sim 50\%$ of the 25 backbone amide groups that are either completely protected with neomycin and completely exposed with kanamycin (or vice versa) are residues having aliphatic hydrocarbon side chains that are buried in hydrophobic patches of the protein, while the other half comprises residues having charged side chains at the surface of the protein.

The protection afforded by AGs in the binary enzyme–AG complexes becomes less effective when the ternary complexes are formed by the addition of MgAMPPCP. A number of backbone amide groups that were completely protected from H/D exchange in the binary complex become exchangeable. This observation, as much as it was totally unexpected, provided the molecular reasons for the observed very large changes in the binding enthalpy of AGs to the enzyme in the presence of metal–ATP[61] and showed that the enthalpic penalty was the result of weakening or breaking of the H-bonds in which these amides are involved. These data thus provided

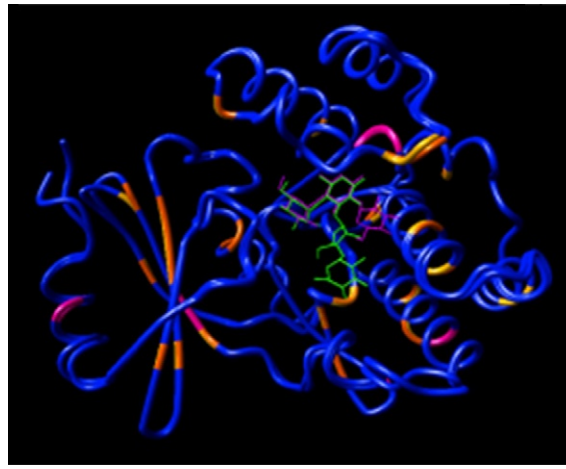

FIG. 12. AG-dependent solvent protection to APH(3′)-IIIa. Amides protected with neomycin only (yellow) and kanamycin only (red) are shown in superimposed structures of the enzyme complexed with both ligands.

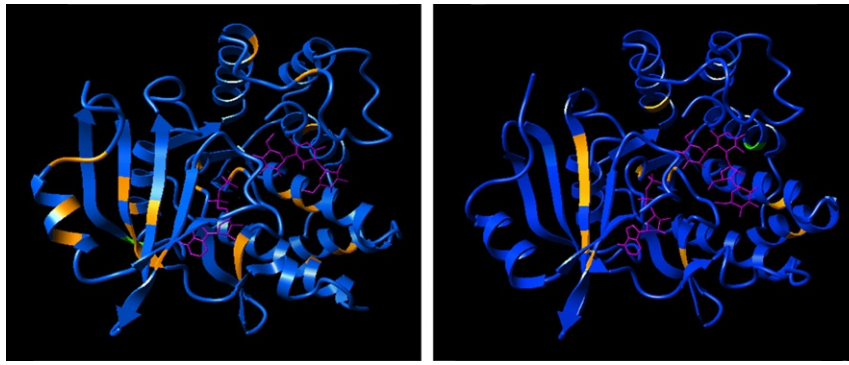

FIG. 13. Residues that become more exposed to solvent (yellow) due to interaction of the nucleotide with APH(3′)-IIIa in complexes of kanamycin (left) and neomycin (right).

a reason for a change in a global thermodynamic parameter and the identity of sites responsible for it. This behavior was, again, AG dependent, and the backbone amide groups that become solvent accessible in the presence of nucleotide are shown in Fig. 13 for the complexes of kanamycin A and neomycin. Overall, these H/D exchange data indicate that the APH(3′)-IIIa "realigns" its conformation from the

core to the surface and alters the dynamic properties of various regions, regardless of their distance to the ligand-binding site, to accommodate structurally different AGs. We note that these changes need not be results of large domain movements or structural arrangements that would cause the formation of a different crystal structure in these complexes but clearly highlight the extremely dynamic nature of this enzyme.

For AAC(3)-IIIb, the promiscuity is not due to overall protein flexibility, as with APH(3′)-IIIa, but instead is the responsibility of a large unstructured loop of $\sim$35 amino acids. AAC(3)-IIIb is a member of the large GNAT superfamily of acetyltransferases that share $<5\%$ overall sequence- similarity but maintain a core fold. This fold includes a conserved, well-structured binding site for acetyl coenzyme A (motif A), while a flexible loop (motif B) is situated at the adjacent site and prepares for association with a molecule to receive the acetylation. This loop has evolved to produce different proteins, each responsible for a unique acetylating function in the cell. An excellent review on GNAT proteins is available.[81] When coenzyme A is pre-complexed with AAC(3)-IIIb, the affinity of the antibiotic increases several fold, thus suggesting that the coenzyme may be inducing a conformational change at the AG-binding site to make binding of antibiotics more thermodynamically favored (ΔH becomes more negative). Molecular dynamics simulations with AAC(3)-IIIb revealed that the loop of motif B is randomly oriented in the apoenzyme; but upon addition of coenzyme A, it undergoes a concerted movement that opens the antibiotic binding site (Fig. 14).[82] This coincides well with the binding of coenzyme A

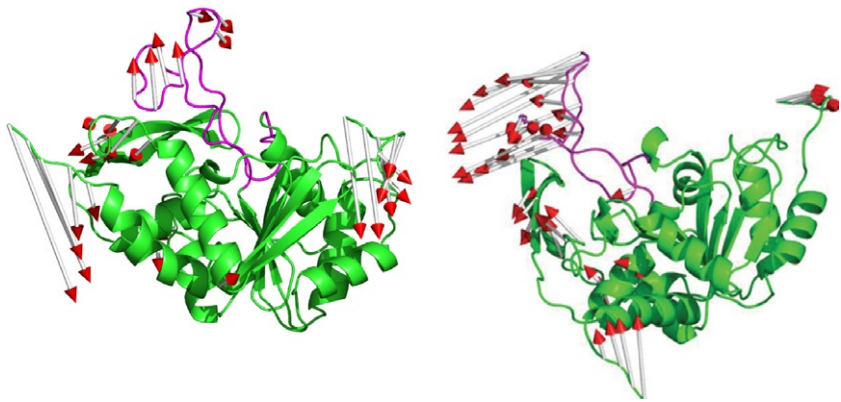

FIG. 14. The flexible, conserved AG-binding loop of AAC(3)-IIIb becomes more flexible upon interaction with CoASH, apoenzyme (left) and enzyme–CoASH complex (right). (Adapted with permission from Ref. 82. Copyright 2011 American Chemical Society).

occurring with a positive entropy and causing shifts of many of the same resonances in the NMR spectrum as antibiotic. The coenzyme A-induced open binding site allows variously structured antibiotics to enter for acetylation, but with an orientation allowing increased favorable interactions between the antibiotic and AAC(3)-IIIb while maintaining 1/1 stoichiometry.

Experimental data supporting the computational studies come from NMR studies. The resonance assignments of AAC(3)-IIIb are not available; however, the ^{15}N–^{1}H HSQC spectrum of the enzyme labeled either with ^{15}N-leucine or ^{15}N-alanine showed that while 28–29 of the expected 29 leucine amide groups are observable, only 30–31 of the expected 39 alanine amide groups could be detected. All leucine residues but one, which is located in the loop, are in well-structured segments of the enzyme. On the other hand, seven alanine residues are located in flexible segments, three are at the N-terminus, and four are in the loop. This information is consistent with the missing number of alanine residues from the spectrum and indicates that these residues are experiencing exchange between two or more conformations over a millisecond range. These properties may be common to GNAT proteins, since the affinity of 2-arylethylamine to arylalkylamine *N*-acetyltransferase increases with coenzyme A.[80]

VI. Conclusions and Future Considerations

Despite any homology in their amino acid sequence, promiscuous AGMEs that show strong overlap in their ligand spectrum appear to use similar strategies to achieve ligand promiscuity. The shared features of ligand–protein interactions include changes in dynamics of the enzyme and its interaction with solvent, both of which are AG dependent. Additionally, the formation of enzyme–AG complexes is rendered thermodynamically favorable by adjusting the binding enthalpy via changes in low-frequency vibrational modes of the protein and its interactions with the solvent. These common molecular properties are compared in greater detail as follows.

First, among three different AGMEs, with no sequence homology between them and catalyzing three different reactions, there are strong, and in some cases very unusual, antibiotic-dependent solvent effects. These are not predictable from the static structures available but underline a fact that the ability to alter interactions with solvent may be needed to achieve substrate promiscuity.

Next, two highly promiscuous AGMEs, namely APH(3′)-IIIa and AAC(3)-IIIb, both have a large unstructured loop involved in AG binding. In AAC(3)-IIIb, the presence of CoASH causes this loop to be more flexible and achieve more-concerted movement. The opposite trends observed in the dependence of ΔH with temperature

between the complexes of this enzyme with neomycin and paromomycin indicate that the binding of paromomycin causes more exposure of hydrophobic surfaces of the protein to solvent, whereas neomycin does the opposite.[83–89] Since ~50% of the residues in the AG-binding loop are those bearing hydrophobic side chains, by adopting different conformations with each AG such changes can easily bring about differential solvent exposure of hydrophobic surfaces. In relation to this change, the loop above the AG-binding site in APH(3′)-IIIa becomes more flexible when the metal–ATP is bound to the enzyme.[78] The H/D exchange patterns of some of the residues on this loop also show AG-dependent differences. For example, Leu-151 and Glu-161 are completely protected in the neomycin complex but are completely exposed in the kanamycin complex. Yet, Glu-160, the residue adjacent to Glu-161, is completely protected with kanamycin A and is exchanged within 41 h with neomycin. The Val-154 residue is protected with both AGs and Leu-147 is exchanged in 12 h with kanamycin A and in 2 h with neomycin. The other residues in this region either exchange quickly or their rates of exchange remain undetermined because of resonance overlap. However, the available data already confirm that this region of the protein adopts different conformations and dynamic properties when complexed with neomycin and kanamycin, causing differential exposure of some amides in this segment. This is also the region of the protein that shows the most significant changes in correlated motions between the kanamycin A- and neomycin-bound APH(3′)-IIIa. It is clear that the increased flexibility in these loops would certainly make it easier to accommodate structurally different AGs. It should also be mentioned that this loop is large in AAC(3)-IIIb and may not need significant changes in other sites, but the smaller loop, as in APH(3′)-IIIa, may need the assistance of changes in other sites as well. Overall, we consider that this is unlikely to be coincidental, and it may represent simple variation in theme rather than a fundamental difference. Thus, flexibility may not only be a common mode of ligand recognition between these two promiscuous AGMEs, but it may also apply to other AGMEs having broad substrate specificity.

It would be interesting to see whether the significant differences observed in the dynamic and thermodynamic properties of enzyme–AG complexes are correlated to their promiscuity. To this end, it might be expected that less-promiscuous enzymes will display less dynamic behavior. Although the accessibility of the active site, or its shape/charge complementarities to AGs, plays a significant role in ligand binding, these factors alone are not sufficient to explain AG-dependent differences in the kinetic, dynamic, and thermodynamic behavior of these enzymes. Therefore, we consider that dynamic properties of these enzymes are the major reason for their substrate promiscuity and have a major impact on their ability to render the binding of structurally diverse AGs thermodynamically favorable. Although the changes

discussed are consequences of ligand binding, we emphasize that protein flexibility is necessary to facilitate them.

Finally, we point out that, in order to have any possibility for development of new antimicrobial agents that are effective against pathogenic bacteria, it is very important to understand those molecular determinants of AG recognition that are common among the enzymes. The development of drugs/inhibitors based on the molecular properties of one enzyme has been unsuccessful.[90] In this way, one target AGME can be inhibited, but the other AGMEs, harbored by bacteria, are not affected, nor is the design of inhibitors based on static structures alone likely to show great success. Studies directed toward understanding the dynamic behavior of these enzymes, and an in-depth understanding of the molecular basis of the AG dependence is needed.

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