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Antibiotic Selection by the Promiscuous Aminoglycoside Acetyltransferase-(3)-IIIb Is Thermodynamically Achieved through the Control of Solvent Rearrangement

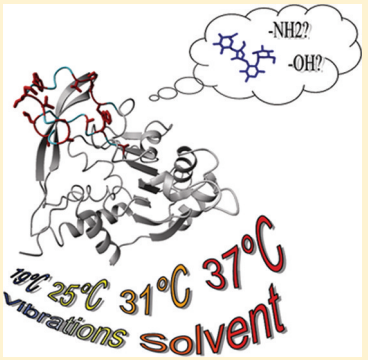
Adrianne L. Norris[†] and Engin H. Serpersu*[,†,‡]

[†]Department of Biochemistry and Cellular and Molecular Biology, The University of Tennessee, Knoxville, Tennessee 37996, United States

[‡]Graduate School of Genome Science and Technology, The University of Tennessee and Oak Ridge National Laboratory, Knoxville, Tennessee 37996, United States, and Bioscience Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States

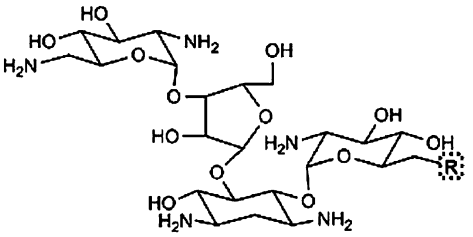
Supporting Information

ABSTRACT: The results presented here show the first known observation of opposite signs of change in heat capacity (ΔC_p) of two structurally similar ligands binding to the same protein site. Neomycin and paromomycin are aminoglycoside antibiotics that are substrates for the resistance-conferring enzyme, the aminoglycoside acetyltransferase-(3)-IIIb (AAC). These antibiotics are identical to one another except at the 6′ position where neomycin has an amine and paromomycin has a hydroxyl. The opposite trends in ΔC_p of binding of these two drugs to AAC suggest a differential exposure of nonpolar amino acid side chains. Nuclear magnetic resonance experiments further demonstrate significantly different changes in AAC upon interaction with neomycin and paromomycin. Experiments in H_2O and D_2O reveal the first observed temperature dependence of solvent and vibrational contributions to ΔC_p. Coenzyme A significantly influences these effects. Together, the data suggest that AAC exploits solvent properties to facilitate favorable thermodynamic selection of antibiotics.



In the complexity of interactions that occur when a protein associates with its ligand(s), changes in solvent structure around solutes can have a dramatic influence on experimentally measured thermodynamic parameters. One such parameter that is thought to be strongly affected by solvent is the change in heat capacity (ΔC_p). Contributions to ΔC_p in various systems have been a topic of much discussion for the past several decades.[1−5] The promiscuous nature of aminoglycoside antibiotic binding proteins provides a unique perspective on this subject. Global properties of interactions between the aminoglycoside acetyltransferase-(3)-IIIb (AAC) and various antibiotics and cofactors have been previously characterized both thermodynamically and kinetically.[6,7] From these, neomycin B and paromomycin were shown to have significant differences in binding affinity, turnover rate, and other thermodynamic parameters. This is interesting because these two antibiotics have identical structures except at one functional group position. Where neomycin boasts an amine (NH_2) group at the 6′ site, paromomycin has a hydroxyl (OH) (Figure 1). Moreover, unlike many other antibiotics, both bind with a 1/1 protein/antibiotic stoichiometry regardless of the presence of the cofactor,[6] thus making this an ideal set of complexes to study further.

This paper describes changes in heat capacity in both H_2O and D_2O of the formation of AAC complexes with neomycin and paromomycin in the absence and presence of coenzyme A. In both solvents, binding of paromomycin has a positive ΔC_p, while for neomycin, the values are negative. This is the first time two ligands so similar in structure and in their mode of binding to the same protein site have shown opposite signs of ΔC_p. Furthermore, association of both neomycin and paromomycin with a preformed complex of AAC and



	R
Neomycin	NH_2
Paromomycin	OH

Figure 1. Similarity of neomycin and paromomycin structures.

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coenzyme A, the noncatalytic form of the required cosubstrate, occurs with equal ΔC_p values, thus supporting the hypothesis that coenzyme A organizes the antibiotic binding site for its optimal interaction. Also, $\Delta\Delta H$ values ($\Delta H_{H_2O} - \Delta H_{D_2O}$) are found to be both complex and temperature-dependent. The correlation between $\Delta\Delta H$ and ΔC_p, a measure of the degree of solvent contribution to a change in heat capacity,[4] is shown to increase with temperature and also varies with the antibiotic and the order of substrate addition. To the best of our knowledge, these are also the first observations of their kind.

AAC is only one protein among many found in bacteria that catalyzes a covalent modification to aminoglycoside-type drugs, thus conferring resistance to the drug's bactericidal action.[8] This system, therefore, not only is of interest to the community of protein−ligand interactions in general but also is strongly clinically important as a representative of aminoglycoside-modifying enzymes and perhaps the large GNAT superfamily of acetyltransferases that are involved in numerous biological processes.[9]

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (99.9%) and [^{15}N]ammonium chloride (99%) were purchased from Cambridge Isotope Laboratories (Andover, MA). Aminoglycosides and all other materials were purchased at the highest possible purity from Sigma-Aldrich.

Protein Overexpression and Purification. AAC was overexpressed in *Escherichia coli* BL21 cells grown in Luria Broth and purified as described previously.[6] For uniformly ^{15}N-labeled AAC, cells were grown in M9 minimal medium enriched with [^{15}N]ammonium chloride to an optical density of 0.8 at 600 nm, induced with 1 mM IPTG for 4.5 h, and purified in the same way as the unlabeled protein. Yields were ∼13 mg of isotopically labeled protein per liter of induced medium.

Isothermal Titration Calorimetry. The change in heat capacity (ΔC_p) was calculated from the linear regression analysis of ΔH versus temperature plots from data obtained at six or more temperatures between 19 and 29 °C. Temperatures above 29 °C gave inconsistent results that correlated well with the activity of the protein, with the activities of various antibiotics decreasing significantly at >30 °C. A few control experiments at lower temperatures were performed and yielded data consistent with the ΔC_p values in the temperature range described above. To maintain uniform pH and protein stability at all experimental temperatures, AAC was dialyzed extensively against a solution of 100 mM NaCl and 50 mM MOPS buffer (pH 7.6) before ITC experiments. The protein concentration in the ITC cell varied between 20 and 35 μM with ligand concentrations in the syringe between 20 and 50 times the AAC concentration, yielding the most accurate ΔH values (c values varied between 2 and 650). For ternary titrations, the cosubstrate concentration present with the enzyme was enough to maintain >98% saturation. The enzymatic activity was found to be >87% of the full activity after ITC experiments. Samples were degassed for 10 min prior to being loaded.

D_2O and H_2O experiments were run in parallel in which samples were identically prepared in H_2O and then the pH was set to 7.6. All samples were then lyophilized to dryness and rehydrated in the proper H_2O or D_2O solvent and incubated at 25 °C for 30−45 min. Incubation for longer periods of time (∼24−48 h) yielded identical results, and the enzymatic activity was not affected by the lyophilization process or the incubation. ITC data collected from a nonlyophilized sample in H_2O yielded results identical to those of the lyophilized sample.

Nuclear Magnetic Resonance. NMR data were collected with uniformly enriched [^{15}N]AAC on a Varian 600 MHz spectrometer equipped with a triple-resonance (^{1}H, ^{13}C, and ^{15}N) cryogenic probe at The University of Tennessee. The protein concentration used was 260 μM in 50 mM MOPS and 100 mM NaCl (pH 7.6) at 29 °C. When applicable, antibiotics were present at concentrations high enough to saturate >98% of the binding sites as calculated from previously determined dissociation constants.[6] Sensitivity-enhanced ^{15}N−^{1}H heteronuclear single-quantum coherence (HSQC) spectra were recorded with 48 scans of 2048 real time points of each of 48 t1 increments with the TROSY option.[10] Spectral widths were 8012 and 2500 Hz for the ^{1}H and ^{15}N dimensions, respectively. Data were processed with nmrPipe[11] where the left half of the spectrum was taken and a sin^2 window function was applied in both dimensions and zero filled to 256 points. No baseline correction or other cosmetic procedures were applied. Data analysis was performed with Sparky (T. D. Goddard and D. G. Kneller, *SPARKY 3*, University of California, San Francisco, CA).

RESULTS AND DISCUSSION

Association of Neomycin and Paromomycin with AAC Occurs with Opposite Changes in Heat Capacity. In previous studies, neomycin was observed to bind 15 times tighter to AAC (K_D = 9.2 ± 0.4 μM) than paromomycin (K_D = 0.6 ± 0.1 μM).[6] On the basis of the relationship $\Delta G = -RT\ln(K_A)$, the affinity differentials create Gibbs energies (ΔG) of AAC's interaction with neomycin and paromomycin of −8.5 ± 0.1 and −6.9 ± 0.04 kcal/mol, respectively. However, intrinsic enthalpies of association (ΔH_{int}) of neomycin and paromomycin with AAC are the same within error.[6] From this, it can be inferred that the dissimilar binding affinities due to an NH_2 to OH substitution may be influencing factors associated with entropy ($-T\Delta S$ values of 7.8 ± 1.0 and 11.5 ± 0.6 kcal/mol for neomycin and paromomycin, respectively). These can include release of water molecules upon binding (more + $T\Delta S$), loss of degrees of freedom of ligand and/or protein (more − $T\Delta S$), or an increase in the degrees of conformational freedom of flexible regions of the protein upon binding ligand (more + $T\Delta S$) in certain cases.[12−15] Of course, combinations of these effects are possible and often likely contributors to entropy changes in any given macromolecule−ligand interaction. Different ligands can also shift the degree to which each factor contributes to an entropy change as observed in the case of ANT(2″) in which neomycin displaced zero net water molecules upon binding while kanamycin caused a net uptake of ∼20 waters.[14,16]

To dissect these further, we determined the temperature dependence of the thermodynamic parameters of all possible AAC−ligand complexes. The change in enthalpy as a function of temperature over short temperature ranges usually remains linear and yields the change in heat capacity (ΔC_p) of the system as a result of the binding event in question. For the binding of neomycin to AAC, ΔC_p is negative, while for paromomycin, the value is positive; each case is accompanied by a compensating change in entropy, thus yielding similar free energy changes at all temperatures (Figure 2 and Table 1).

Although heat capacity changes of protein−ligand interactions have been studied for some time, to the best of our knowledge, no system ever demonstrated an opposite sign of ΔC_p for two ligands that bind to the same site on the protein and are so similar in structure. The most related literature

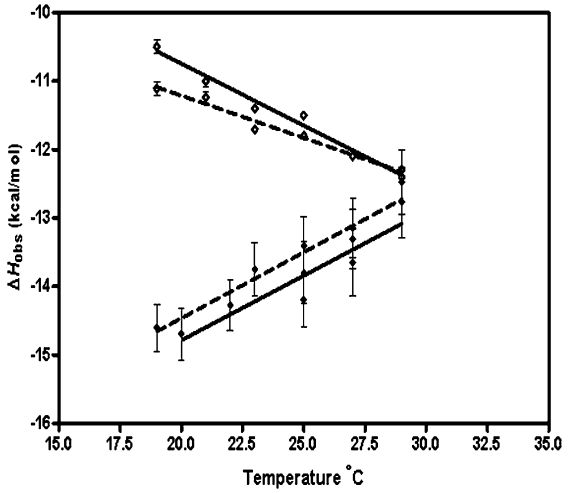

Figure 2. Heat capacity changes for association of paromomycin and neomycin with AAC. Solid lines show data from experiments in H_2O, while dashed lines show data for experiments in D_2O: neomycin (◇) and paromomycin (◆). Error bars represent the standard error of the mean from two to three independent trials.

Table 1. Solvent Specific Heat Capacity Changes of Neomycin and Paromomycin Complexes[a]

AAC complex	$\Delta C_{p(H_2O)}$	$\Delta C_{p(D_2O)}$	$\Delta C_{p(tr)}$
neomycin binary	−0.18 ± 0.00	−0.12 ± 0.01	−0.06 ± 0.01
paromomycin binary	0.19 ± 0.00	0.19 ± 0.03	0.00 ± 0.02
neomycin ternary	−0.35 ± 0.04	−0.20 ± 0.05	−0.15 ± 0.05
paromomycin ternary	−0.32 ± 0.03	−0.14 ± 0.02	−0.18 ± 0.03
CoASH(N)	−0.19 ± 0.02	−0.12 ± 0.01	−0.07 ± 0.02
CoASH(P)	−0.29 ± 0.03	−0.31 ± 0.03	0.02 ± 0.03

[a]Units are in kilocalories per mole per kelvin. Ternary complexes were formed by titration of the antibiotic to a preformed complex of AAC with CoASH. (N) and (P) denote neomycin and paromomycin, respectively, precomplexed to AAC prior to CoASH titration. Errors are from the standard error of the mean from two or three independent trials.

discussion can be found in recent studies of the N-terminal ATP binding domain of the Hsp90 protein.[17] In this case, AMPPNP and an inhibitor, geldanamycin (GA), bind to the same site of Hsp90 but are very structurally dissimilar to one another. Here, binding showed a slightly positive heat capacity change for AMPPNP and a negative change for GA. These opposite trends were accredited to a differential Hsp90 conformation while it was bound to each ligand in which there was a change in the degree of exposure of hydrophobic side chains to solvent. It is also noteworthy that GA, with its negative ΔC_p, was reported to bind to Hsp90 with >10-fold higher affinity than AMPPNP.

Our observations with AAC indicate that significant changes in ΔC_p in response to the binding of different aminoglycosides to the enzyme appear to be a hallmark of aminoglycoside-modifying enzymes (AGMEs). Another highly promiscuous AGME, the aminoglycoside phosphotransferase-(3′)-IIIa (APH), shows a dramatically different ΔC_p between the kanamycin- and neomycin-bound forms; the binary APH−kanamycin complex shows a highly temperature dependent heat capacity change even over a short range of temperatures, while the neomycin complex has a temperature-independent ΔC_p.[18] One difference between the two enzymes is that absolute ΔC_p values observed for AAC−aminoglycoside interactions are within the range observed for most carbohydrate−protein interactions (−0.1 to −0.7 kcal mol^{-1} K^{-1}),[19] while they were significantly more negative for aminoglycoside−APH interactions (−0.7 to −3.8 kcal mol^{-1} K^{-1}).[18] In the case of APH, such large changes in heat capacity are attributed to a highly dynamic apoenzyme that gains a well-defined structure upon antibiotic association.[13] Data thus far collected for AAC suggest that the flexible antibiotic binding loop responds the most to aminoglycosides while the rest of the protein remains fairly unaltered.[6,7] Such an observation correlates well with the differences in the magnitude of ΔC_p between these two aminoglycoside-modifying enzymes.

The Sign of ΔC_p for AAC−Ligand Complexes Implicates Differential Exposure of Nonpolar Residues. Exposure or burial of hydrophobic groups upon binding of a ligand to a protein is one event thought to significantly contribute to the sign of ΔC_p.[2−4,20−22] In proteins, the ΔH associated with transfer of polar and nonpolar moieties from the protein core to solvent includes enthalpies of breaking hydrogen bonds and van der Waals interactions, respectively, as well as an enthalpy of hydration.[23] The enthalpy of hydration of polar and nonpolar groups is negative at low temperatures but changes in opposite directions with an increase in temperature.[23,24] Moreover, the total enthalpy of transfer of nonpolar groups increases in magnitude and goes through zero between 18 and 25 °C, while the enthalpy of polar groups becomes more negative with an increase in temperature;[23,25] thus, ΔC_p will be opposite in sign for such transfers. It is reasonable then that if two ligands induce different conformational changes in the protein in which hydrophobic groups are differentially exposed or buried, opposite signs of ΔC_p values will be observed. Because the 6′-amine group of neomycin will be protonated at the experimental pH of 7.6 (the pK_a of unbound neomycin is 8.1), it is likely an electrostatic interaction, not possible with the hydroxyl of paromomycin, is the initiator of the proposed differential structures of the AAC−neomycin and AAC−paromomycin complexes. A more detailed discussion of the possible contributions that a change in the pK_a of the 6′-amine group might make to ΔC_p is provided below.

Crystal structures of AAC from which a change in solvent accessible surface area could be calculated for neomycin and paromomycin complexes relative to apo-AAC are not available at this time. However, a differential exposure or burial of nonpolar amino acids is logical because 50% of the residues in the large loop predicted to be part of the aminoglycoside binding site have hydrophobic side chains (Figure 3). Neomycin and paromomycin might cause this loop to undergo relatively different changes in its structure, either directly or from propagated effects, which would result in a differential hydration state of its residues and hence ΔC_p. Again these differences can likely be attributed to the extra positive charge expected to be present at the 6′ position of neomycin. From this, the positive ΔC_p accompanying paromomycin binding suggests a net exposure of hydrophobic groups, while the negative ΔC_p of neomycin association is net burial. Furthermore, the change in entropy (ΔS) of binding of paromomycin to AAC is less favorable than that of neomycin.[6] Water molecules involved in hydration shells around nonpolar side chains will have a lower entropy than those of bulk solvent. Therefore, if paromomycin is indeed inducing an increase in solvent-exposed nonpolar surface area while neomycin is not, entropy would be more negative for formation of the former protein−antibiotic complex as observed.

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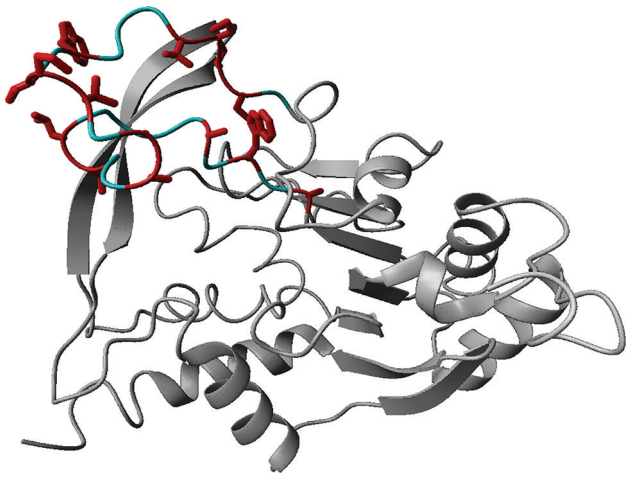

Figure 3. Nonpolar residues on the antibiotic binding loop. The antibiotic binding loop (light blue) is comprised of several residues with nonpolar side chains (red). This structure is the from the previously published homology model.[6]

It has previously been established that formation of the ternary complexes via antibiotic titration (i.e., association of an antibiotic with a preformed complex of AAC and CoASH) is the most enthalpically favored and entropically disfavored method of all those of the complexes studied.[6,7] Like in binary complex formation, ΔH values are similar within error for paromomycin and neomycin ternary complexes while $T\Delta S$ is more unfavorable for that of paromomycin. Neomycin binds 15-fold tighter to AAC than paromomycin and 37-fold tighter to the AAC−CoASH complex.[6] This suggests that tighter binding ligands do not necessarily imply that a larger number of bonds are formed relative to a weaker binding ligand with the same protein. It is well-known that ligand-induced conformational changes in a protein beyond the active site can contribute significantly to the changes in the enthalpy and entropy of the system. An expanded region of the HSQC spectra acquired with different complexes of AAC, shown in Figure 4, reveals a change in the chemical environment of some backbone amides dependent on whether AAC is bound to neomycin (blue peaks) or paromomycin (green peaks). This occurs in a large number

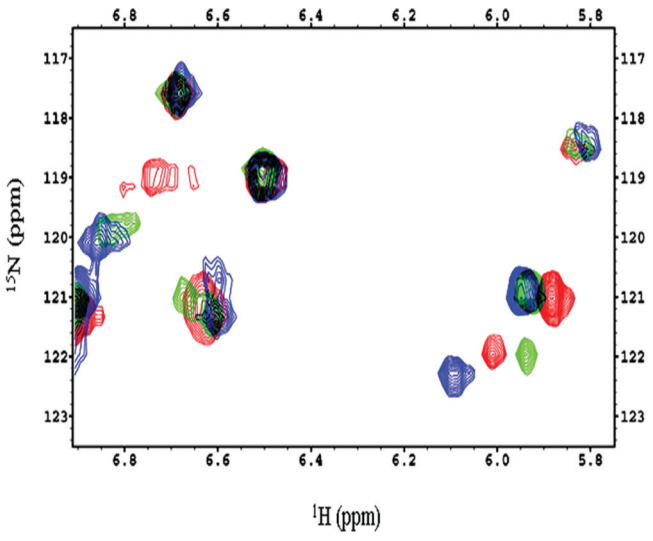

Figure 4. ^{15}N−^{1}H correlation spectra for AAC−apo (red), AAC−paromomycin (green), and AAC−neomycin (blue) complexes.

of residues (>30) in the entire spectrum, thus supporting the hypothesis that residues beyond the locale of the 6′ position are feeling differential effects from these two antibiotics. Figure 1 of the Supporting Information shows an overlay of the full NMR spectra in which purple arrows highlight some of the regions showing similar changes as in the expanded view in Figure 4.

Contrary to what is observed in the formation of binary enzyme−aminoglycoside complexes, ΔC_p values for the binding of neomycin and paromomycin to the binary AAC−CoASH complex to form the respective ternary complexes are (a) negative and (b) similar to each other (Table 1). This is in excellent agreement with the hypothesis that CoASH reorganizes the antibiotic binding site to make aminoglycoside interaction most optimal, and subsequent interaction with these structurally comparable antibiotics occurs with similar changes in heat capacity. The hypothesis can now include the suggestion that when the cosubstrate site is occupied by CoASH, a net burial of nonpolar groups occurs with both antibiotics.

Heat capacity changes for the reverse titrations, i.e., association of CoASH with an AAC−antibiotic complex, are also negative. However, the magnitude of ΔC_p is different for association with AAC−neomycin and AAC−paromomycin complexes, where the latter is more negative [Table 1, CoASH(N) and CoASH(P), respectively]. It is logical that paromomycin, having prearranged AAC's conformation to have a net exposure of nonpolar residues, would create a situation in which the binding of CoASH would then have more nonpolar groups to bury compared to the neomycin-bound AAC. This explains the more negative change in heat capacity for association of CoASH with the AAC−paromomycin versus AAC−neomycin complex.

Other factors can also potentially contribute to a change in heat capacity. Eftink and colleagues have suggested that proteins, in the absence of ligands, sample multiple interconverting conformational "microstates" in which ligand binding can shift the equilibrium to a more uniform population with an energy lower than that of the mixed microstate population.[26] This would be observed as a decreased (more negative) ΔC_p of the system. Let us consider that the antibiotic binding loop of AAC is indeed flexible and is a source of multiple conformational states in the apoprotein. One might imagine that the significantly tighter binding of neomycin can cause the loop to be "held in place" while with paromomycin it can still shift between a subset of conformations in which temperature may influence the equilibrium between them. This might explain why the heat capacity change for association of paromomycin is less negative than that of neomycin but cannot be fully attributed to the fact that it is positive. Indeed, a positive ΔC_p would be observed if paromomycin causes an increase in the number of conformational states that AAC undergoes relative to unliganded AAC. These correlate well with the negative ΔC_p values for ternary complexes and suggests that conformational equilibria, at least in certain regions of AAC, could also contribute to heat capacity changes. In relation to this, several decades ago, Sturtevant discussed contributions to heat capacity changes in terms of hydrophobic hydration as well as internal vibrational motions of proteins, hydrogen bonds, and electrostatic effects.[3] From these, it was suggested that the hydrophobic effect and changes in vibrational modes will have the strongest influence on changes in heat capacity such that $\Delta C_p = \Delta C_{p(\mathrm{hydro})} + \Delta C_{p(\mathrm{vib})}$. In his equations, contributions from temperature-dependent conformational equilibria to ΔC_p are neglected, which likely does not apply to AAC−ligand interactions. However, if we use his method of calculation to determine contributions of $\Delta C_{p(\mathrm{hydro})}$

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and $\Delta C_{p(vib)}$ to the overall ΔC_p, the binary AAC−paromomycin complex becomes the only outlier among all six complexes, which qualitatively agrees with the other complex-dependent trends we observed.

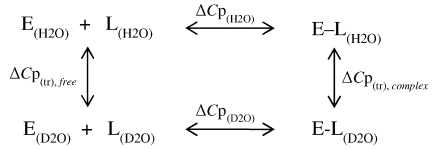
Finally, the heat of ionization associated with a shift in the pK_a values of the titratable groups on the ligand and/or protein upon binding may be affected by temperature increases and thus also contribute to ΔC_p. Neomycin has one more amine than paromomycin, and the pK_a of this group on unbound neomycin is known to be 8.1.[27] If the binding of neomycin to AAC causes shifts of amine pK_a values to higher values as observed with APH,[27] then one can assume that these shifts can contribute to the observed ΔC_p. In that case, an upshift in the pK_a of the 6′-amine upon binding of neomycin to AAC to cause a 100% protonation from NH_2 to NH_3^+ would decrease ΔC_p by only ∼9.2 cal mol^{-1} K^{-1}.[23] However, at the experimental pH (7.6), this amine group will be >68% protonated at the beginning; therefore, a change in the protonation state of the ligand is not expected to be a major contributor to either the sign or the magnitude of ΔC_p for the neomycin-bound versus paromomycin-bound complexes of AAC. On the other hand, ionizable groups on AAC may also undergo a change in protonation state upon binding. Earlier, we observed that binding of neomycin to AAC occurs with a net uptake of 0.6 proton, while with paromomycin, the net uptake is 1.1 protons.[6] This net uptake includes protonation from both ligand and protein and again shows a small difference between the two antibiotics. These numbers are not significantly different for ternary complexes.[6,7] Together, they rule out changes in protonation as a major contributor to the observed differential heat capacities and strongly suggest that the specific interaction of the groups at the 6′-site with the enzyme is responsible for the observed differences between the complexes of these two aminoglycosides. This finding is further strengthened by the fact that the presence of a positive charge versus a hydroxyl group at this site does have an only small impact on the overall charge of the molecule because there five other positively charged amines are present on both molecules.

Solvent Isotope Effect. The discussion included above points mainly to an antibiotic-dependent solvent structure in AAC complexes contributing to ΔC_p, although changes in protein vibrational modes and conformational equilibria may also be influencing it. Changing the solvent from H_2O to D_2O and determining thermodynamic parameters will provide information that is associated with changes in solvent structure to help differentiate contributions to ΔC_p. Light and heavy water are biochemically very similar, but slight differences such as hydrogen bond formation enthalpies (∼10% more favored in D_2O)[28,29] and internal vibrational frequencies (higher for D_2O by ∼10%)[30,31] allow insight into solvent interaction properties within the system in question. Such data allow calculation of $\Delta\Delta H$ as $\Delta H_{H_2O} - \Delta H_{D_2O}$. Chervenak and Toone defined any observed ΔH of a protein−ligand interaction as the sum of the intrinsic enthalpy of binding (ΔH_{int}), i.e., non-solvent-related enthalpy, and the enthalpy of solvent reorganization resulting from the binding event (ΔH_s), such that $\Delta H_{obs} = \Delta H_{int} + \Delta H_s$.[4] As ΔH_{int} is the same in H_2O and D_2O, any non-zero $\Delta\Delta H$ would have to arise because of differential ΔH_s values. A negative $\Delta\Delta H$ value means that the event producing a change in the enthalpy of the system is more favored in H_2O than it is in D_2O. For a ligand's association with an enzyme, the sign of $\Delta\Delta H$ is often discussed in terms of stability in which D_2O is differentially stabilizing the free and bound states.

To date, few solvent studies deriving $\Delta\Delta H$ have been conducted on protein−ligand interactions.[4,18,20,32] In all but one case, $\Delta\Delta H$ is a negative value. The exception is another aminoglycoside-modifying enzyme, APH, for which kanamycin class antibiotics yield negative values and neomycins yield positive values at 25 °C.[18] At this same temperature, the association of neomycin with AAC has a positive $\Delta\Delta H$, while for paromomycin, the value is negative. This implies that these two aminoglycoside-modifying enzymes, although having a largely overlapping substrate profile, may not undergo the same changes in solvent rearrangement upon binding antibiotics. APH and AAC have no sequence homology and do not share any known structural similarities, so this result is not surprising. However, it suggests a general property of AGMEs: solvent plays a significant role in the recognition of aminoglycoside antibiotics by these enzymes and renders formation of enzyme−ligand complexes thermodynamically favorable for a wide variety of structurally diverse aminoglycosides.

For a few protein−ligand interactions, heat capacity changes were determined in both H_2O and D_2O.[4,20] In all but one instance, ΔC_p was shown to be the same in H_2O and D_2O, where ΔH versus temperature plots were parallel. However, the magnitudes of ΔH in H_2O and D_2O were different, yielding a negative $\Delta\Delta H$ that remained constant at all temperatures. Moreover, an approximately linear correlation was observed between $\Delta\Delta H$ and ΔC_p for all of these systems, which was interpreted to mean that solvent rearrangement (the only factor significantly influencing a non-zero $\Delta\Delta H$) must be the dominant contributor to the observed heat capacity changes for those systems.[4] It was postulated that a nonlinear correlation would imply that a change in the low-frequency vibrational modes of the enzyme or other factors must dominate. To the best of our knowledge, the only protein−ligand interaction showing a differential heat capacity change in H_2O and D_2O is from the work of Connelly and colleagues, who studied the association of rapamycin with FK506 and briefly suggested that this could be due to differential heat capacities of hydration of polar groups.[20]

In our system, the sign of ΔC_p for the formation of any given complex is the same in H_2O and D_2O (i.e., that for formation of the AAC−paromomycin complex is positive, while those for all other complexes are negative in both solvents). Therefore, all previous discussion about contributions to the sign of ΔC_p is still valid. However, the signs and magnitudes of $\Delta\Delta H$ values are found to be both complex and temperature-dependent. To the best of our knowledge, this is the first such observation of its kind. A plot of $\Delta\Delta H$ versus temperature represents the change in heat capacity upon transfer of the specified enzyme−ligand complex from H_2O to D_2O [$\Delta C_{p(tr)}$] (Figure 5 and Table 1). Consider the following scheme:



where $\Delta C_{p(H_2O)} - \Delta C_{p(D_2O)} = \Delta C_{p(tr)} = \Delta C_{p(tr),complex} - \Delta C_{p(tr),free}$. It follows that a negative $\Delta C_{p(tr)}$ means that the heat capacity change of transferring the complex is more negative than that of the free enzyme and ligand. Studies by Lopez and Makhatadze on model compounds have shown that the ΔC_p is negative for the transfer of polar surfaces from H_2O to D_2O and positive for nonpolar surfaces.[33] Of the six complexes studied,

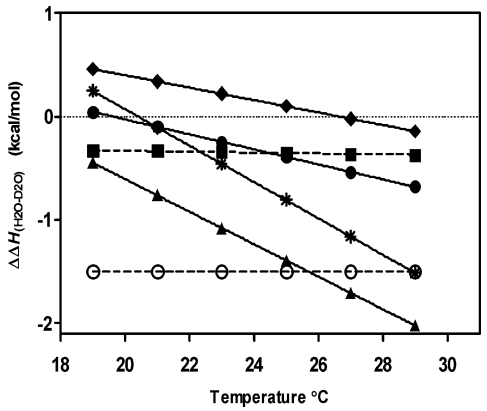


Figure 5. Temperature dependence of $\Delta\Delta H$: (◆) neomycin binary, (∗) paromomycin ternary, (●) CoASH(N), (■) paromomycin binary, (▲) neomycin ternary, and (○) CoASH(P) complexes, with (N) and (P) representing neomycin and paromomycin, respectively, as the antibiotic precomplexed with AAC prior to CoASH association. Data for the two complexes with zero slopes, paromomycin binary and CoASH(P), are shown with dashed lines for the sake of clarity. All errors are ≤10% from two or three independent trials.

the binary AAC−paromomycin complex and the ternary complex formed by the titration of the binary AAC−paromomycin complex with CoASH showed $\Delta C_{p(tr)}$ values of ≈0. For all other complexes, values are differentially negative. This means that when AAC is in the latter complexes, there is more solvent accessible polar surface area than in the free enzyme. This is also consistent with the sign of each determined ΔC_p being negative, which indicates burial of hydrophobic surfaces and/or exposure of polar surfaces as previously discussed.

Formation of ternary complexes via titration of either paromomycin or neomycin to the binary AAC−CoASH complex occurs with similar $\Delta C_{p(tr)}$ values. This is in excellent agreement with the hypothesis that CoASH "arranges" the antibiotic site for optimal antibiotic interaction. Moreover, these complexes show the strongest magnitude of $\Delta C_{p(tr)}$; thus, these must have the most polar surfaces exposed among all complexes studied (Figure 5 and Table 1). As mentioned, the $\Delta C_{p(tr)}$ values are not equivalent for the CoASH(N) and CoASH(P) complexes. Altogether, these data support the hypothesis that paromomycin alters the structure of AAC in a manner different from that of neomycin and that CoASH association does not recompense the degree of change.

$\Delta\Delta H$ values for the four complexes that showed a temperature-dependent sign reversal between 16 and 27 °C (Figure 5) indicate that below these temperatures the formation of these complexes is more favored in D_2O. Although it is known that the transfer of polar groups from H_2O to D_2O occurs with a negative heat capacity change, there is a discrepancy in the sign of $\Delta H_{(tr)}$ given in literature for various polar groups where some showed positive $\Delta H_{(tr)}$ values[34,35] or nonpositive $\Delta H_{(tr)}$ values.[33,36,37] All data are given for the measurements taken at 25 °C, which is in the middle of the range of crossover temperatures determined in our experiments, and are close to zero. This may explain why some of the enthalpies of transfer were positive while some were negative. Thus, our experiments emphatically suggest that the sign of $\Delta\Delta H$ for a protein−ligand interaction must be interpreted with caution as the temperature can have a significant effect on both the sign and the magnitude of $\Delta\Delta H$. While model studies are good references from which to understand the trends of common moieties, fully understanding the behavior of a given protein−ligand complex and its subsequent solvent rearrangement properties requires determination of $\Delta\Delta H$ at a variety of temperatures if the system allows.

Dissection of the Temperature Dependence of $\Delta\Delta H$.

Data shown thus far support the hypothesis that paromomycin and neomycin are causing AAC to form different conformations and may imply that the structure of AAC is altered depending upon the order in which substrates are bound. For now, let us exclude the AAC−paromomycin complex and the association of CoASH with the AAC−paromomycin complex as these two do not demonstrate a $\Delta\Delta H$ temperature dependence. As mentioned previously, for a given series of interactions for which $\Delta\Delta H$ and ΔC_p are measured, a linear correlation between the two is suggested to reflect the fact that solvent rearrangement is the dominating contributor to heat capacity changes in those systems. This was based on data acquired with different protein−ligand interactions.[4] In our case, all the complexes being studied include the same protein. However, because of the temperature-dependent nature of $\Delta\Delta H$, such a plot had to be generated at each experimental temperature (six total points). Here it was observed that at low temperatures, the correlation coefficient (R^2) is quite low (∼0.4) but increases systematically until it reaches a saturation level close to 1.0 (a perfect correlation) (Figure 6). This means that at lower temperatures, vibrational and other non-solvent-related phenomena are contributing more to ΔC_p than solvent,

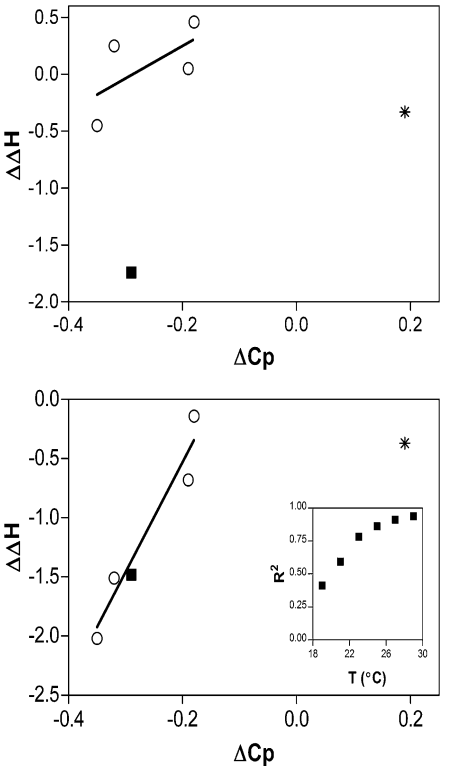


Figure 6. $\Delta\Delta H$ vs ΔC_p plots of AAC−ligand complexes. The top panel shows data at 19 °C, while the bottom panel shows data at 29 °C: (■) CoASH(P), (∗) paromomycin binary, and (○) neomycin binary, neomycin ternary, CoASH(N), and paromomycin ternary complexes. The inset shows that the correlation coefficient (R^2) of the (○) complexes systematically increases from a weak correlation (∼0.4) to an almost perfect correlation (∼0.95).

but solvent effects progressively contribute more with an increasing temperature. Let us now consider the two "outlier"

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complexes, paromomycin binary and CoASH(P) ternary. On a plot of $\Delta\Delta H$ versus ΔC_p, the positions of these two complexes will not move with an increase in temperature while the positions of all other complexes do.

As shown in Figure 6, while the temperature increases and the R^2 value moves progressively closer to 1.0 for the four other complexes, their positions in the plot get closer and closer to forming a linear correlation with the CoASH(P) ternary complex. This suggests that either this is coincidental or solvent effects are the dominant contributor to ΔC_p for the CoASH(P) ternary complexes at all temperatures studied. The binary AAC−paromomycin complex now remains as the only outlier if the observation with the CoASH(P) complex described above is not coincidental. It is not easy to explain this behavior because the sign of ΔC_p for the binary AAC−paromomycin complex is positive, indicating the exposure of hydrophobic groups to solvent. Obviously, this will cause solvent effects to contribute to the observed ΔC_p. One may suggest that solvent effects are present but not dominant in this complex in the temperature range studied. The magnitude of opposing contributions could offset the solvent effect, hence leaving ΔC_p unchanged as with the CoASH(P) complex but preventing it from falling into a linear correlation with the other complexes. It is also possible that the CoASH(P) and AAC−paromomycin complexes are both outliers in the $\Delta\Delta H$ versus ΔC_p plots. Here, solvent effects may dominate the ΔC_p in both complexes, but AAC's conformation may be different from those of the other complexes because of association of paromomycin without or before CoASH interaction. Our data are not sufficient to dissect this phenomenon at this time.

Other Possible Contributors to $\Delta\Delta H$. Other factors may contribute to a heat of transfer, as well. First, exchangeable hydrogen atoms on solutes will be replaced with deuterium. Slightly shorter and stronger bonds are formed with deuterium than with hydrogen, thus contributing to differential enthalpy changes between these systems.[28,29,38] AAC, being comprised of 274 amino acids, will have many exchangeable hydrogens, while neomycin and paromomycin have relatively few. AAC−ligand complexes are not expected to have a significantly different number of exchangeable hydrogens compared to free protein with free ligand. As $\Delta\Delta H$ is the heat of transfer of the complex minus the heats of transfer of free ligand and protein, it is expected that heats from hydrogen exchange will make very little contribution to $\Delta\Delta H$. NMR-detected hydrogen−deuterium exchange experiments show that the AAC−neomycin and AAC−paromomycin complexes have a similar number of exchangeable backbone amide hydrogens (between 15 and 18% of observable residues remain unexchanged after exposure to D_2O for 76 h) and only slightly more than apo-AAC where ∼11% are unexchanged (A. L. Norris and E. H. Serpersu, unpublished data), thus supporting this assumption.

It is known that the pK_a values of functional groups differ by 0.4 pH unit in the two solvents, and hence, differential protonation states of both ligand and protein can occur. This also results in unequal concentrations of free D^+ and H^+ in the two solvents. However, it has been shown that the 0.4 unit difference in the pH meter readings in D_2O and H_2O solutions directly offsets this effect.[39] Experiments in which pH = pD are therefore expected to yield similar protonation states for their solute functional groups, thus canceling out any associated heat of transfer.

CONCLUSIONS

Results from this work reveal the first example of two ligands that are almost identical in structure and bind to the same enzymatic site that induces opposite signs for the ΔC_p of binding. Paromomycin and neomycin, differing only at the 6′ position (Figure 1), bind with a positive and negative ΔC_p values, respectively. Furthermore, for the CoASH(P) complex with AAC in which paromomycin is bound first, solvent rearrangement appears to be the dominant contributor to ΔC_p at all temperatures studied. With the paromomycin binary complex, solvent must be contributing to ΔC_p in accordance with its positive sign in both H_2O and D_2O, indicative of exposure of hydrophobic side chains to solvent. In this case, other factors may be contributing to mask the expected linear correlation of $\Delta\Delta H$ with ΔC_p as observed with all other complexes at 29 °C. In these latter complexes, vibrational and/or other non-solvent-related phenomena dominate at lower temperatures, while the solvent progressively contributes more as the temperature increases. To the best of our knowledge, this observation is also the first of its kind. As AAC had a large number of hydrophobic amino acids, many of which are situated at the antibiotic binding loop, these differences are likely related to nonpolar side chains of AAC residues being exposed or buried upon formation of the different ligand complexes where the degree of exposure and/or burial is correlated with temperature. On the basis of our results, it can be suggested that $\Delta\Delta H$ experiments performed at only one temperature can be misleading, and a complete understanding of a given protein−ligand interaction would require such data at multiple temperatures.

Formation of the ternary complexes by addition of an antibiotic to a preformed complex of AAC and coenzyme A yields similar values of ΔC_p and $\Delta\Delta H$ for both paromomycin and neomycin binding. The reverse is not true, thus supporting our hypothesis that coenzyme A arranges the antibiotic binding site to make aminoglycoside interaction most thermodynamically favorable. Solvent structures and protein vibrational modes are therefore similarly affected by paromomycin and neomycin when coenzyme A is present. However, in its absence, these two drugs adjust said parameters differentially. Discernment of the exact effect on AAC's structure by paromomycin and neomycin will require more detailed structural studies. However, in conjunction with previously known heat capacity and solvent data for APH, a promiscuous aminoglycoside phosphotransferase, it can be said that interactions of ligand with these antibiotic resistance enzymes are significantly influenced by solvent. Differential rearrangement of solvent allows the formation of enzyme−antibiotic complexes with structurally diverse antibiotics to be favorable through enthalpic contributions. This may be a general property of all aminoglycoside-modifying enzymes and the reason for their broad substrate promiscuity.

ASSOCIATED CONTENT

Supporting Information

NMR spectrum of uniformly ^{15}N-enriched AAC with purple arrows highlighting some of the regions showing similar changes as in the expanded view in Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Walters Life Sciences Bldg. M407, The University of Tennessee, Knoxville, TN 37996-0840. Telephone: (865) 974-2668. Fax: (865) 974-6306. E-mail: serpersu@utk.edu.

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■ ABBREVIATIONS

AAC, aminoglycoside acetyltransferase; ΔC_p, heat capacity change; GNAT, GCN5-related acetyltransferase; IPTG, isopropyl β-D-1-thiogalactopyranoside; PMSF, phenylmethanesulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; 2-DOS, 2-deoxystreptamine; AG, aminoglycoside; AGME, aminoglycoside-modifying enzyme; CoASH, coenzyme A; ITC, isothermal titration calorimetry; HSQC, heteronuclear single-quantum coherence; APH, aminoglycoside phosphotransferase-(3′)-IIIa.

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