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Solvent Reorganization Plays a Temperature-Dependent Role in Antibiotic Selection by a Thermostable Aminoglycoside Nucleotidyltransferase-4′

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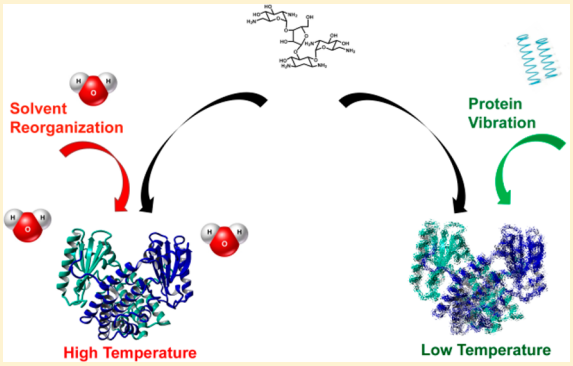
Supporting Information

ABSTRACT: The aminoglycoside nucleotidyltransferase-4′ (ANT) is an enzyme that causes resistance to a large number of aminoglycoside antibiotics by nucleotidylation of the 4′-site on these antibiotics. The effect of solvent reorganization on enzyme-ligand interactions was investigated using a thermophilic variant of the enzyme resulting from a single-site mutation (T130K). Data showed that the binding of aminoglycosides to ANT causes exposure of polar groups to solvent. However, solvent reorganization becomes the major contributor to the enthalpy of the formation of enzyme−aminoglycoside complexes only above 20 °C. The change in heat capacity (ΔC_p) shows an aminoglycoside-dependent pattern such that it correlates with the affinity of the ligand for the enzyme. Differences in ΔC_p values determined in H_2O and D_2O also correlated with the ligand affinity. The temperature-dependent increase in the offset temperature (T_{off}), the temperature difference required to observe equal enthalpies in both solvents, is also dependent on the binding affinity of the ligand, and the steepest increase was observed with the tightest binding aminoglycoside, neomycin. Overall, these data, together with earlier observations with a different enzyme, the aminoglycoside *N*3-acetyltransferase-IIIb [Norris, A. L., and Serpersu, E. H. (2011) *Biochemistry 50*, 9309], show that solvent reorganization or changes in soft vibrational modes of the protein are interchangeable with respect to the role of being the major contributor to complex formation depending on temperature. These data suggest that such effects may more generally apply to enzyme−ligand interactions, and studies at a single temperature may provide only a part of the whole picture of thermodynamics of enzyme−ligand interactions.



The aminoglycoside nucleotidyltransferase-4′ (ANT) is an enzyme that causes resistance to a large number of aminoglycoside antibiotics by nucleotidylation of the 4′-site on aminoglycoside antibiotics (Figure 1). The crystal structures of the D80Y variant bound to kanamycin A and AMPCPP[1] and a double mutant (D80Y/T130K) in apo form are available.[2] The active form of the enzyme is a homodimer. Each monomer binds both substrates, MgATP and aminoglycoside; however, the reaction occurs between substrates that are bound to different subunits (Figure 2).[1] Previous studies performed with a thermostable variant of ANT generated by a single mutation (T130K) showed that the enzyme is in a monomer−dimer equilibrium and binding of aminoglycosides, but not MgATP, shifts the equilibrium toward dimer formation as detected by analytical ultracentrifugation.[3] Thermodynamic studies showed that the enzyme can bind a large number of aminoglycosides with favorable enthalpy and unfavorable entropy.[3]

Heat capacity is one of the major thermodynamic quantities that are measured in proteins and protein−ligand interactions. The sign of the change in heat capacity (ΔC_p) informs on solvation of polar and nonpolar groups. Multiple sources can also affect ΔC_p; exposure of hydrophobic surfaces to solvent, changes in soft vibrational modes of proteins, and conformational changes may be the more important sources.[4] In this work, we investigated the effect of solvent reorganization in recognition of aminoglycosides by ANT. Contrary to many observations reported in the literature, where the exposure or burial of nonpolar surfaces usually determines the effects of solvent on the thermodynamics of protein−ligand interactions, we observed that exposure of polar groups of ANT to solvent dominates the thermodynamics of protein−ligand interactions. However, the role of solvent reorganization in the enthalpy of ligand binding shows a strong temperature dependence and becomes a dominant factor only above 20 °C.

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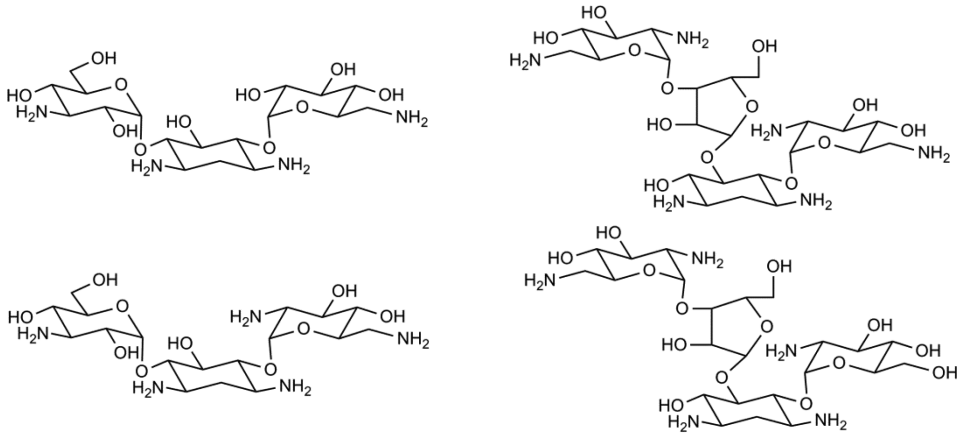

Figure 1. Chemical structures of aminoglycoside antibiotics: kanamycin A (top left), tobramycin (bottom left), neomycin B (top right), and paromomycin (bottom right).

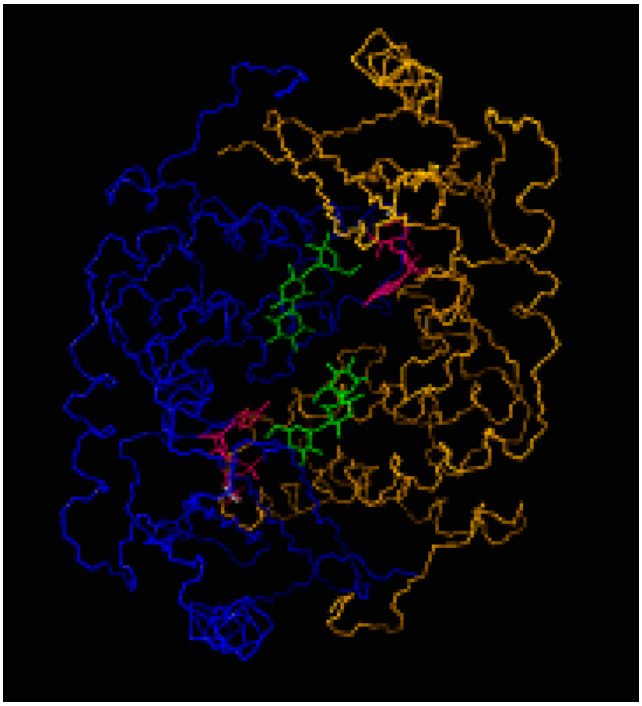

Figure 2. Crystal structure of ANT. Two monomer subunits are colored blue and yellow. Bound kanamycin A molecules are colored green and Mg-AMPCPP molecules magenta (Protein Data Bank entry 1KNY).[1]

MATERIALS AND METHODS

Reagents. All materials were of the highest purity commercially available and were purchased from Sigma-Aldrich Co. (St. Louis, MO) unless otherwise noted. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Ultrafiltration membranes were purchased from Millipore (Billerica, MA). Isopropyl β-D-1-thiogalactopyranoside was obtained from Inalco Spa (Milan, Italy), and high-performance Ni-Sepharose resin was purchased from GE Healthcare (Pittsburgh, PA). Aminoglycosides were purchased as sulfate salts and used only as a base after the removal of sulfate ion via ion exchange chromatography.

Protein Expression and Purification. A thermostable variant of ANT, T130K, was purified as described previously.[3] Purified ANT was dialyzed against buffer containing 50 mM MOPS (pH 7.5) and 100 mM NaCl. For D_2O experiments, enzyme in H_2O was buffer-exchanged with D_2O buffer containing 50 mM MOPS (pD 7.5, >98% D_2O) and 100 mM NaCl using five cycles of ultrafiltration. Purified ANT in H_2O and D_2O was stored at 4 °C, and the enzyme remained active (>90%) for 3 weeks. Protein concentrations were determined by the absorbance at 280 nm using an extinction coefficient of 50880 M^{-1} cm^{-1}. Protein concentrations are reported as monomer concentrations unless otherwise indicated.

Isothermal Titration Calorimetry. ITC experiments were performed over a temperature range of 5−40 °C using a VP-ITC microcalorimeter from (formerly Microcal) GE Healthcare. Because the uncertainty in ligand concentration is a major contributor to the errors in ITC data, in our laboratory, concentrations of aminoglycoside antibiotics were are always determined by enzymatic assays or by nuclear magnetic resonance.[5] In this case, we used another aminoglycoside-modifying enzyme, the aminoglycoside *N*3-acetyltransferase-IIIb, which has high turnover rates.[6] Concentrations of AGs are determined by five or six repetitions, and errors ranged between 1 and 3% for different aminoglycosides and are included in the data analysis as experimental error.

Isothermal titration calorimetry (ITC) experiments were performed as described previously with other aminoglycoside-modifying enzymes.[6−10] The concentration of ANT in the calorimetry cell was 20 μM (monomer). The aminoglycoside concentration in the syringe was in the range of 0.2−0.8 mM. Both enzyme and ligand solutions were degassed under vacuum for 15 min prior to the start of experiments. A buffer system composed of 50 mM MOPS (pH 7.5) and 100 mM NaCl was used in all titration experiments. Titrations consisted of 27 injections of 10 μL and were separated by 240 s. A cell stirring speed of 300 rpm was used. The observed heat change (ΔH) evolving from binary complex formation and the binding affinity (K_a) were directly determined from titration. Gibbs energy (ΔG) and entropy ($T\Delta S$) changes associated with binding were determined from eqs 1 and 2.

$$\Delta G = -RT \ln K_a \quad (1)$$

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

Origin version 5.0 was used for nonlinear least-squares fitting of binding data to a single-site binding model. Data were also fit by using global fitting procedure SEDPHAT.[11] Errors shown in

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plots are standard errors of the mean (SEM) based on two or more repetitions of the titrations of most of the data points and represent experimental errors rather than curve fitting errors.

RESULTS

Aminoglycosides Show Differential Effects on the Change in Heat Capacity of ANT. The temperature dependence of the binding enthalpy for the binding of four aminoglycosides to ANT was measured to determine the change in heat capacity in H_2O and D_2O. Two aminoglycosides from the kanamycin group (kanamycin A and tobramycin) and two from the neomycin group (neomycin and paromomycin) were used (Figure 1). Figure 3 shows data acquired with two of

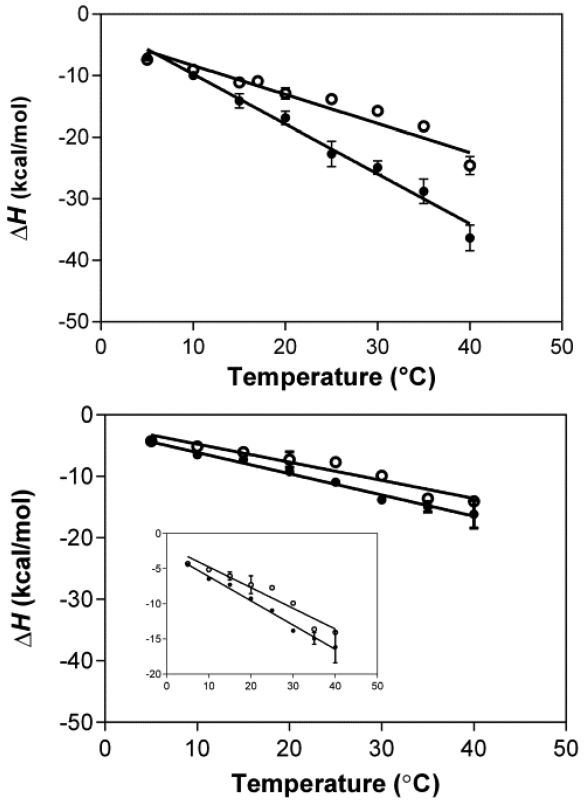

Figure 3. Temperature dependence of binding enthalpy. Binding of neomycin (top) and kanamycin A (bottom) to ANT in D_2O (○) and H_2O (●). Data are shown with linear regression lines. Error bars represent the standard error of mean from two or three independent trials. The inset shows an expanded view of the kanamycin A data.

the four aminoglycosides that showed the largest (neomycin) and smallest differences (kanamycin A) in binding enthalpies determined in light and heavy water. The heat capacity change (ΔC_p), determined from the slopes of plots of ΔH versus temperature, was negative in all four cases but was dependent on the aminoglycoside (Table 1). With the exception of kanamycin A, all ΔC_p values, determined in H_2O, are significantly more negative than those observed in most carbohydrate−protein interactions, which is usually between 0.1 and 0.4 kcal mol^{-1} K^{-1}. The determined ΔC_p values showed a logarithmic correlation with the binding affinity of the aminoglycoside to the enzyme in both solvents (Figure 4), indicating a direct correlation between the Gibbs energy and the heat capacity change.

Table 1. Solvent Specific Heat Capacity Changes of ANT−AG Complexes[a]

	$\Delta C_p(H_2O)$	$\Delta C_p(D_2O)$	$\Delta C_{p,tr}$[b]
neomycin	−0.81 ± 0.06	−0.47 ± 0.05	0.34 ± 0.06
paromomycin	−0.67 ± 0.05	−0.41 ± 0.05	0.26 ± 0.05
tobramycin	−0.55 ± 0.01	−0.39 ± 0.04	0.16 ± 0.04
kanamycin A	−0.35 ± 0.03	−0.29 ± 0.03	0.06 ± 0.03

[a]Units are in kilocalories per mole per degree Celcius. Data shown with fitting errors from plots of ΔH vs T. [b]$\Delta C_{p,tr} = \Delta C_p(D_2O) - \Delta C_p(H_2O)$.

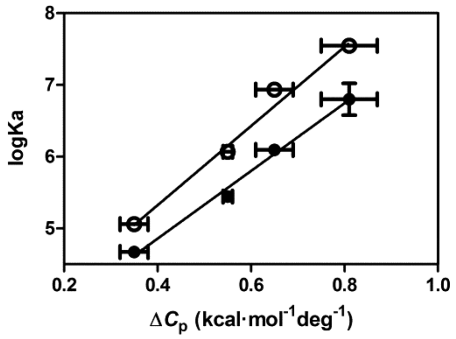

Figure 4. Binding constants (log K_a) for the binding of aminoglycosides to ANT as a function of the heat capacity change in H_2O. Each data point represents a different enzyme−aminoglycoside complex at 25 °C (○) and 40 °C (●). Error bars of log K_a are the standard error of mean from two or three independent trials.

Binding Enthalpies in H_2O and D_2O Are Aminoglycoside-Dependent. Binding of all aminoglycosides was enthalpically more favored in H_2O than in D_2O above 10 °C. Differences in binding enthalpies of aminoglycosides ($\Delta H_{H_2O} - \Delta H_{D_2O} = \Delta\Delta H_{H_2O-D_2O}$, henceforth $\Delta\Delta H$), determined from the slopes of plots of ΔH versus temperature (Figure 3), are displayed in Figure 5 as a function of temperature. These data

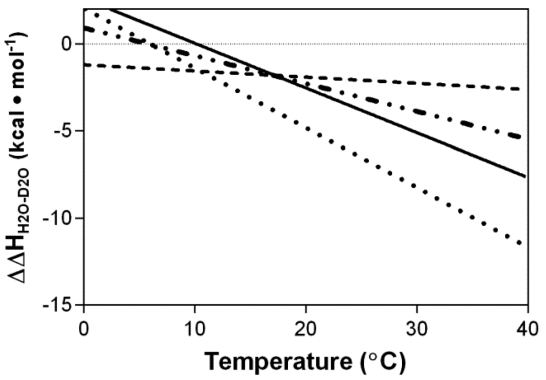

Figure 5. Temperature dependence of $\Delta\Delta H$ of ANT−aminoglycoside complexes: neomycin (···), paromomycin (—), tobramycin (—··—), and kanamycin A (---). $\Delta\Delta H$ values were determined from the slopes of linear regression lines from plots of ΔH vs T (Figure 3).

show that binding enthalpy becomes more exothermic in D_2O below the range of 5−10 °C with the exception of that of kanamycin A. If neomycin, which is by far the tightest binding antibiotic to ANT, is excluded, all lines intersect at 17.2 ± 0.2 °C. ΔC_p was almost identical in H_2O and D_2O for kanamycin A, while it was more negative in H_2O with all others. Differences between the determined ΔC_p in H_2O and D_2O

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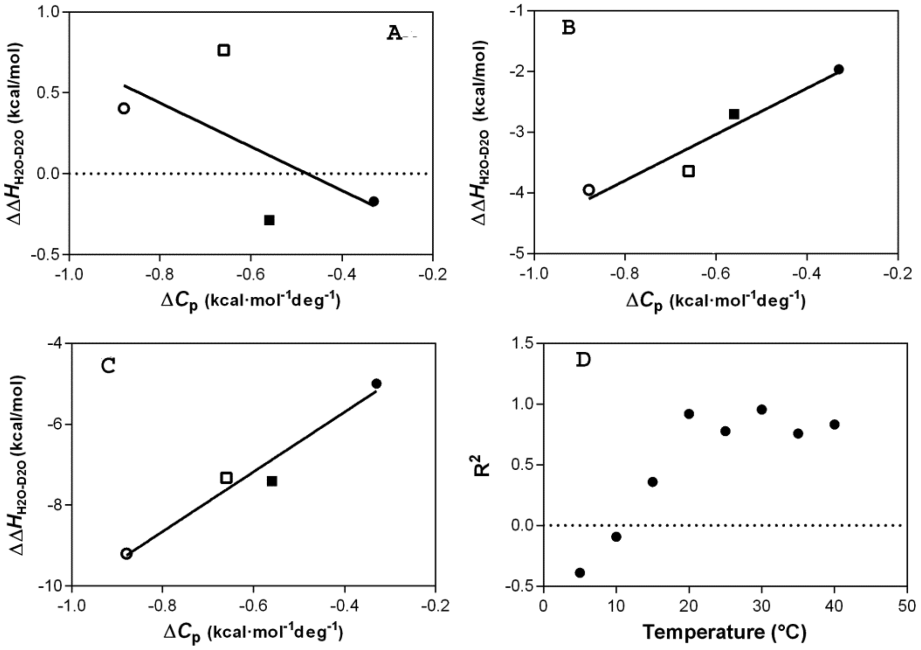

Figure 6. Plots of $\Delta\Delta H$ vs ΔC_p of ANT−ligand complexes. Panels A−C show data at 5, 20, and 40 °C, respectively, for neomycin (○), paromomycin (□), tobramycin (■), and kanamycin A (●). Data are shown with regression lines. Panel D shows the linear correlation coefficient (R^2) of the plots as a function of temperature.

($\Delta C_{p,H_2O} - \Delta C_{p,D_2O} = \Delta\Delta C_p$) were aminoglycoside-dependent; the largest $\Delta\Delta C_p$ was observed with neomycin, while $\Delta\Delta C_p$ was almost zero for kanamycin A. $\Delta\Delta C_p$ values also correlated well with the binding affinity of the aminoglycoside for the enzyme; the largest $\Delta\Delta C_p$ was observed with the tightest binding aminoglycoside neomycin, and $\Delta\Delta C_p$ was ∼0 for the weakest binding kanamycin A (Table 1).

The Effect of Solvent Reorganization on Heat Capacity Is Temperature-Dependent. The enthalpic difference in H_2O and D_2O ($\Delta\Delta H$) was taken at different temperatures for ANT−aminoglycoside complexes and plotted versus ΔC_p. A good linear correlation in plots of $\Delta\Delta H$ versus ΔC_p shows that the solvent reorganization is the main contributor to binding enthalpy.[12] The slope of such plots has units of temperature and represents the offset temperature (T_{off}), which is the temperature difference needed to observe identical enthalpies of binding in light and heavy water. When such plots were made for ANT−aminoglycoside complexes, there was a strong dependence on the temperature such that the correlation increased linearly with an increasing temperature, reaching to a plateau above 20 °C. Examples of such plots at three different temperatures are shown in Figure 6A−C. The dependence of the correlation coefficient on temperature is shown in Figure 6D.

DISCUSSION

Changes in Heat Capacity. One of the major factors affecting ΔC_p for ligand−protein complexes is the exposure or burial of hydrophobic and polar surfaces.[4,13−15] Hydration of polar and nonpolar groups is negative at low temperatures and changes in opposite directions with an increasing temperature such that it becomes more negative with polar groups and less negative with nonpolar groups, crossing into positive values in the temperature range of 18−25 °C.[16,17] Data acquired with ANT using four different aminoglycosides indicate that the major contribution to the change in heat capacity is the change in the interaction of polar groups with water (solvent rearrangement). The negative values of ΔC_p correlated well with the binding affinity of ligands for ANT, yielding the most negative value with the tightest binding neomycin (Table 1). These data are consistent with the protein becoming "stiffer" with the binding of ligands, which correlates well with the previously published data acquired during analytical centrifugation studies as well as the observed negative ΔC_p values.[3]

The binding of aminoglycosides to ANT occurs with changes in the net protonation of titratable groups on the ligand and the protein.[3] Therefore, we have always used multiple buffers with different heats of ionization to perform such a titration to account for the contribution of the buffer to the observed enthalpy and to determine the intrinsic enthalpy.[3,6,8−10,18] In this case, changes in temperature may cause differential shifts in pK_a values of these groups and contribute to the observed ΔC_p. However, differences in the temperature dependence of buffer pK_a values would complicate the data analysis. Therefore, we used a single buffer (MOPS) with the lowest temperature-dependent pK_a. Also, such effects are not likely to be the main cause of differences observed between the determined ΔC_p values for different enzyme−aminoglycoside complexes because even 100% protonation of NH_2 to NH_3^+ would decrease ΔC_p by only ∼9.2 cal mol^{-1} deg^{-1},[16] which is too small to account for the observed differences despite the fact that there are four to six amine groups present in the aminoglycosides used in this work (Figure 1). We should also note that pH versus pD differences in H_2O and D_2O are usually compensated for proteins in these solvents.[19]

Solvent Isotope Effect. Solvent reorganization and changes in vibrational and conformational modes of protein are usually believed to be the major contributors to ΔC_p.[4,20] The relative magnitude of each contribution has long been a source of debate. The plot of $\Delta\Delta H$ versus ΔC_p has been previously used to test the contribution of solvent reorganization to ΔC_p.[7,12] A linear correlation between $\Delta\Delta H$ and ΔC_p is

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indicative of a major contribution from solvent reorganization and a minor contribution from changes in protein dynamics. To determine the contribution of solvent reorganization to ΔC_p, we investigated binding of aminoglycosides to ANT in H_2O and D_2O as solvents. There are only a few cases in which heat capacity changes were determined in both solvents. In these studies, it was observed that ΔC_p can be different in both solvents or remain the same. ANT−aminoglycoside interactions showed a strong temperature dependence, and at low temperatures, no correlation exists between $\Delta\Delta H$ and ΔC_p. Contrary to this case, at higher temperatures there is a very strong correlation. These data indicate that changes in heat capacity mostly reflect the effects of solvent reorganization above 20 °C. At lower temperatures, it is likely that a change in soft vibrational modes of the protein may become the more dominant contributor to the change in heat capacity.[4,7,12] Because ΔC_p shows a linear correlation with log K_a, the association constant for the enzyme−aminoglycoside complex, these observations suggest that the major contributor to ΔC_p may also be the major contributor to ΔG for complex formation. This is consistent with solvent reorganization being the major contributor to formation of the complex between the enzyme and aminoglycosides above 20 °C.

Most studies, described in the literature, have been performed at 25 °C only. The first study showing that the effect of solvent reorganization on binding enthalpy can be temperature-dependent was performed with the aminoglycoside *N*3-acetyltransferase-IIIb (AAC), for which the linear correlation between $\Delta\Delta H$ and ΔC_p increased with an increasing temperature.[7] This work describes the observation of such an effect for the second time. Although both AAC and ANT are members of a large family that modify aminoglycoside antibiotics, they catalyze different reactions and have no significant sequence or structural homology to each other, yet solvent reorganization contributes to the change in heat capacity in a temperature-dependent manner with the formation of enzyme−aminoglycoside complexes for both enzymes. These observations suggest that the temperature dependence of solvent reorganization may be more general than specific for these two enzymes but has not been investigated previously. These findings also demonstrate that different factors may become dominant as a function of temperature, and such dependence may have other consequences in terms of protein−ligand interactions at different temperature regimes.

It is likely that the role of water molecules and their interaction with the ligand and the enzyme may be different with each aminoglycoside and may alter the correlation between the change in heat capacity and $\Delta\Delta H$ at different temperatures. Variations in $\Delta\Delta H$ for each aminoglycoside can be due to either different exposures of polar groups or the spatial distribution of water in the active site.[21] The lack of a crystal structure with different aminoglycosides precludes us from making this distinction; however, it is likely that both factors contribute to the observed differences between different aminoglycosides. Interactions with solvent may have a significant impact on carbohydrate conformation,[22] and if conformational fluctuations of aminoglycosides favor different conformations with temperature variations, interactions with solvent may be different and alter the energetics of desolvation and binding at different temperatures. To this end, earlier work showed that aminoglycosides can bind the aminoglycoside-modifying enzymes in multiple conformations.[23−26] Because the strong temperature dependence of water reorientation in hydrophobic hydration shells has been described previously,[27] our data suggest that reorientation of water on polar surfaces may also be dependent on temperature.

The enthalpy of transfer from H_2O to D_2O ($\Delta H_{tr} = \Delta H_{D_2O} - \Delta H_{H_2O}$) has been reported to be slightly negative or close to zero[28,29] or positive[21,30] for polar groups at 25 °C. Our results show that ΔH_{tr} becomes more positive with an increase in temperature. Data shown in Figure 7 are determined from the

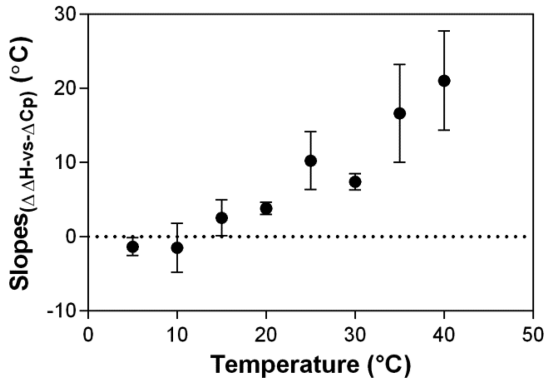

Figure 7. Slopes of plots $\Delta\Delta H$ vs ΔC_p. Data are shown as a function of temperature.

slopes of plots of $\Delta\Delta H$ versus ΔC_p (Figure 5) and demonstrate the progressive increase in T_{off} with an increase in temperature. This indicates that at higher temperatures the temperature difference for obtaining equal binding enthalpy in water and heavy water becomes larger. The offset temperature observed in other systems at 25 °C yielded values such that they are reminiscent of other offsets between H_2O and D_2O attributed to different hydrogen bonding strengths.[12] These include boiling points (1.4 K), critical points (3.8 K), melting points (4 K), triple points (4 K), and temperatures of maximal density (8 K). Our results with another AGME, the aminoglycoside *N*3-acetyltransferase-IIIb also fall in the same range.[7] However, results of this work support the notion that this need not be the case. With ANT, differences are in this range only in the low-temperature regime, and as the temperature increases from 25 to 40 °C, T_{off} is found to systematically increase and exceed the T_{off} range that was observed in previous studies. In our case, T_{off} is around 10 K for 25 °C and increases to 20 K at 40 °C. These data suggest that T_{off} is not a simple representation of differences between physical properties of light and heavy water but may include contributions from other properties of the enzyme and/or the ligand.

The difference in the binding enthalpy of a ligand in H_2O and D_2O is thought to represent the enthalpy of transfer of part of the ligand and enzyme active site that is desolvated during binding.[31] This difference in enthalpy is also interpreted as the measure of enthalpy available through desolvation of the ligand and the protein.[21] It is interesting to note that the difference becomes larger at higher temperatures for this thermophilic variant of ANT and directly correlated with the affinity of the ligand for the protein such that it is largest with the tightest binding neomycin. We should also note that among these four aminoglycosides, the two tightest binding ligands are also the two largest, neomycin and paromomycin. Because T_{off} increases faster with an increasing temperature for these two aminoglycosides, it is reasonable to assume that the largest surface

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area may need to be desolvated on the enzyme and the ligand. On the contrary, the sign of $\Delta\Delta H$ and its variation with temperature were different even with structurally almost identical aminoglycosides with AAC.[7]

The crystal structure of ANT with bound kanamycin A shows that the antibiotic is completely surrounded by polar side chains. A close-up view of the active site is shown in Figure 8,

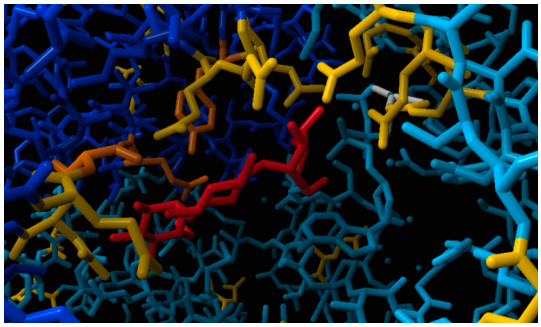

Figure 8. Close-up view of the ANT active site.[1] Kanamycin A (red) is completely surrounded by polar side chains. Carboxylic side chains are colored yellow and other polar side chains orange.

where the carboxylic side chains are colored yellow and other polar side chains orange. A ΔC_p for the transfer of NH_2/NH_3^+ groups from H_2O to D_2O above 25 °C exists.[13] The thermodynamic data listed in Table 1 are also consistent with this structure.

Finally, plots of ΔH versus $T\Delta S$ showed variable slopes as a function of temperature (Figures S1 and S2 of the Supporting Information). Although slopes of plots of ΔH versus ΔS were significantly different from the temperature of the experiment, the data are acquired with only four ligands and quite noisy. Therefore, no firm interpretations can be made; however, it is tempting to suggest that the balance of enthalpic and entropic contributions to complex formation may also be dependent on temperature.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

AG, aminoglycoside; AGME, aminoglycoside-modifying enzyme; ANT, aminoglycoside nucleotidyltransferase-4′; ITC, isothermal titration calorimetry; MOPS, 3-(*N*-morpholino)-propanesulfonic acid.

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