# Detection of Specific Solvent Rearrangement Regions of an Enzyme: NMR and ITC Studies with Aminoglycoside Phosphotransferase(3′)-IIIa†

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ABSTRACT: This work describes differential effects of solvent in complexes of the aminoglycoside phosphotransferase(3′)-IIIa (APH) with different aminoglycosides and the detection of change in solvent structure at specific sites away from substrates. Binding of kanamycins to APH occurs with a larger negative $\Delta H$ in $H_2O$ relative to $D_2O$ ($\Delta\Delta H_{(H_2O-D_2O)} < 0$), while the reverse is true for neomycins. Unusually large negative $\Delta C_p$ values were observed for binding of aminoglycosides to APH. $\Delta C_p$ for the APH−neomycin complex was −1.6 kcal·mol$^{-1}$·deg$^{-1}$. A break at 30 °C was observed in the APH−kanamycin complex yielding $\Delta C_p$ values of −0.7 kcal·mol$^{-1}$·deg$^{-1}$ and −3.8 kcal·mol$^{-1}$·deg$^{-1}$ below and above 30 °C, respectively. Neither the change in accessible surface area (ΔASA) nor contributions from heats of ionization were sufficient to explain the large negative $\Delta C_p$ values. Most significantly, $^{15}N-^{1}H$ HSQC experiments showed that temperature-dependent shifts of the backbone amide protons of Leu 88, Ser 91, Cys 98, and Leu143 revealed a break at 30 °C only in the APH−kanamycin complex in spectra collected between 21 °C and 38 °C. These amino acids represent *solvent reorganization sites* that experience a change in solvent structure in their immediate environment as structurally different ligands bind to the enzyme. These residues were away from the substrate binding site and distributed in three hydrophobic patches in APH. Overall, our results show that a large number of factors affect $\Delta C_p$ and binding of structurally different ligand groups cause different solvent structure in the active site as well as differentially affecting specific sites away from the ligand binding site.

Aminoglycosides are a large group of antibiotics. They exert their bactericidal effect by interfering with translational fidelity of protein synthesis (*1*−*5*). The majority of aminoglycosides belong to the 2-deoxystreptamine (2-DOS) structural group which is composed of a cyclitol ring with amino sugars connected to it by glycosidic bonds. Figure 1 shows structures of two major classes of aminocyclitols, kanamycins and neomycins.

Bactericidal effectiveness of aminoglycosides was severely reduced in recent decades due to the emergence of bacterial strains that are resistant to their action. A large number of enzymes produced by these bacteria can acetylate, nucleotidylate, or phosphorylate the aminoglycoside antibiotics and eliminate their effects as antibacterial agents. The aminoglycoside phosphotransferase(3′)-IIIa (APH) is one of the most widespread resistance enzymes in the aminoglycoside phosphotransferase family of modification enzymes. It carries out MgATP-dependent O-phosphorylation of the 3′-OH or 5″-OH of aminoglycosides. Because of its wide substrate selectivity, APH has been the subject of various structural, kinetic, and mechanistic studies (*6*−*11*). Thermodynamic studies of APH−ligand complexes showed that aminoglycoside−APH association was enthalpically favored and entropically disfavored (*12*, *13*), which is typical for carbohydrate−protein interactions (*14*, *15*). Even thermodynamic parameters of structurally similar aminoglycosides show significant differences, which implies that certain functional groups of aminoglycosides have important roles in recognition of aminoglycosides by aminoglycoside-modifying enzymes (*12*, *13*, *16*, *17*) consistent with earlier kinetic data obtained with substrate analogues (*18*). However, detailed analysis of thermodynamic parameters of enzyme−aminoglycoside association was not feasible because of contribution of multiple processes such as changes in p$K_a$ values of several groups on aminoglycosides as well as the enzyme upon formation of the enzyme−aminoglycoside complex. Therefore, we began dissecting enzyme−aminoglycoside interactions by determining contributions of individual sites to the global thermodynamic properties of enzyme−ligand complexes. To this end, we determined p$K_a$s of the amino groups in enzyme-bound neomycin (*12*). This work represented the first determination of p$K_a$s in a target-bound aminoglycoside and allowed us to determine contribu-

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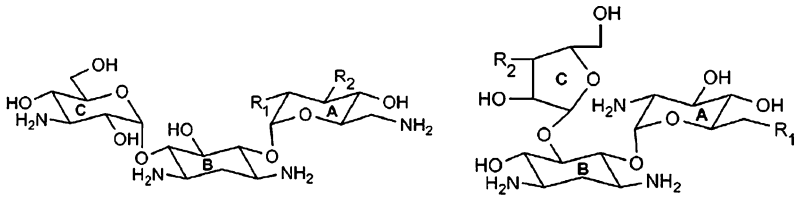


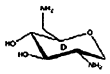
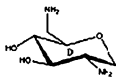
| | $R_1$ | $R_2$ | | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| Kanamycin A | OH | OH | Ribostamycin | $NH_2$ | OH |
| Kanamycin B | $NH_2$ | OH | Neomycin | $NH_2$ |  |
| Tobramycin | $NH_2$ | H | paromomycin | OH |  |

FIGURE 1: Structures of selected aminoglycosides.

tions of p$K_a$ changes to the global thermodynamic parameters. The current paper represents our continued effort to dissect aminoglycoside−enzyme interactions in order to understand roles of individual contributions from the ligand and the enzyme in determining thermodynamic properties of APH−aminoglycoside complexes.

Results described in this paper show that not only does the solvent play a significant role in the formation of the enzyme−aminoglycoside complex but it may have specific effects in binding of different classes of aminoglycosides to the enzyme. Furthermore, an unusually large negative change in heat capacity accompanies binding of aminoglycosides to the enzyme with an apparent break in the binary enzyme−kanamycin complex. Our results also showed that a similar break was detectable in the temperature-dependent chemical shift patterns of four residues on the enzyme in the APH−kanamycin complex, which may represent *solvent reorganization sites* on the enzyme that are away from the ligand binding site.

## EXPERIMENTAL PROCEDURES

*Materials.* The aminoglycoside phosphotransferase(3′)-IIIa (APH) was purified as described earlier (*8*, *13*). Purified APH was concentrated using Millipore (Billerica, MA) ultrafiltration membranes and stored at −80 °C. DTT was purchased from Inalco Spa (Milano, Italy). Aminoglycoside antibiotics and all other general chemicals were obtained from Sigma (St. Louis, MO). $D_2O$ and $^{15}N$-enriched salts were purchased from Cambridge Isotope Laboratories (Andover, MA).

*Isothermal Titration Calorimetry.* Binding of aminoglycosides to APH was determined by ITC[1] titrations performed as described earlier (*13*). Briefly, these experiments were conducted using a VP-ITC microcalorimeter from Microcal, Inc. (Northampton, MA). Concentration of APH in the calorimetry sample cell was 20 $\mu$M. Aminoglycoside concentration in the syringe was in the 0.4 to 0.5 mM range. A buffer system composed of 50 mM HEPES, pH 7.2, and 10 mM DTT (freshly added to the enzyme solution and incubated 1 h at 24 °C before titration) was used in all experiments. Sodium ion concentration was adjusted to 100 mM using NaCl. $H_2O/D_2O$ experiments were carried out at 24 °C. Uncorrected pH-meter readings were used. In a few control experiments pD was adjusted according to pD = pH + 0.4. The pH of samples used in measurement of $\Delta C_p$ was also adjusted at each temperature. Samples were degassed under a vacuum for 10 min before loading to the cell and syringe of the calorimeter. Each titration experiment was set for 29 injections of 10 $\mu$L of ligand solution into the sample cell with 240 s separation. Stirring speed was set to 300 rpm.

Origin software package (v0.5) was used for nonlinear least-squares fitting of binding data to a single site binding model. We should note that in a recent study, largely representing a repetition of a part of our earlier work (*13*) by simply replacing one MgATP analogue (CaATP) with another (MgAMPPNP), Pilch and co-workers were unable to fit their data to a single site model for APH−neomycin complex but used a two-site model with affinities differing by about 2 orders of magnitude (*19*). Unfortunately, these researchers ran into pitfalls of sample preparation, data analysis, and interpretation that we have already described in an earlier paper (*12*). One of these issues is the strong tendency of this enzyme to form dimers in solution, even under mildly reducing conditions (*8*). The down side of the dimer formation is that it alters substrate binding and creates two sites with different affinities to aminoglycosides. To this end, binding of neomycin to the dimeric APH showed two binding sites with 3 orders of magnitude difference in their affinity to the ligand such that a break became clearly visible in the thermograms and fitted data (*12*). Affinity of neomycin to monomeric APH is about 10-fold lower than the high affinity site of the dimer, falling between the two sites of the dimer. From the conditions used by the Pilch group, it is clear that they worked with a mixture of monomeric and dimeric forms of the enzyme. Thus, it is not surprising that their data did not fit to a single site model.

The formation of dimeric APH occurs via an intermolecular disulfide bond between Cys 156 of one molecule to Cys 19 of the second. This bond does not exist under physiological conditions where the enzyme is monomeric (*20*). Cys 156 is in the region of residues (147−170) that adopt different conformations in the presence and the absence of aminoglycosides (*10*). Involvement of Cys 156 in an

[1] Abbreviations: HSQC, heteronuclear single quantum coherence; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ITC, isothermal titration calorimetry.

intermolecular disulfide bond would obviously affect the conformation of this segment which also explains why aminoglycosides show different affinities to each site on the dimer. Therefore, we strongly emphasize on maintaining the monomeric state of the enzyme under all experimental conditions, which is always confirmed with HPLC. In addition, we do not rely only on fitted calorimetric data for stoichiometry of enzyme−aminoglycoside complexes. NMR titrations, performed with different aminoglycosides by following changes in the $^{15}$N−$^{1}$H-HSQC spectrum of $^{15}$N-enriched APH clearly showed that once the 1/1 complex of aminoglycoside/enzyme was formed by the presence of slight excess of an aminoglycoside, no changes in the spectrum were observed by further additions of aminoglycosides up to four to 5-fold excess of the enzyme. This is consistent with binding to a single site under the conditions of all ITC experiments.

*NMR Spectroscopy*. NMR spectra were acquired on a 600 MHz Varian INOVA spectrometer equipped with a triple-resonance probe using uniformly $^{15}$N-enriched APH. NMR samples contained 250 $\mu$M $^{15}$N-enriched APH in 50 mM HEPES buffer, pH 7.5, with 350 $\mu$M kanamycin A or 300 $\mu$M neomycin B (henceforth both aminoglycosides will be referred as kanamycin and neomycin, respectively) to ensure at least a 95% saturation of the binding sites. DTT was also present at 10 mM concentration to prevent dimerization of the enzyme. Sensitivity-enhanced $^{1}$H−$^{15}$N HSQC spectra (*21*) with the TROSY option (*22*) were acquired in the phase-sensitive mode using the States−Haberkorn method for quadrature detection in the indirect dimension (*23*). Datasets were obtained with a spectral width of 8012 Hz in the $^{1}$H dimension and 2500 Hz in the $^{15}$N dimension and 32−64 scans of 2048 real time points for each of 80 t1 increments were recorded. The data were processed using the Felix processing software package (Accelerys, San Diego, CA) and displayed using either NMRview (*24*) or Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco) software. The $^{15}$N dimension was zero-filled to 256 points, with the sensitivity enhancement option selected on the left half of the spectrum. No baseline correction or other cosmetic procedures were applied.

## RESULTS

*Effect of Solvent on Binding of Aminoglycosides to APH.* The contribution of solvent to the enthalpy of carbohydrate−protein complexes can be significant. Therefore, we determined thermodynamic parameters of APH−aminoglycoside complexes in $H_2O$ and $D_2O$ by isothermal titration calorimetry to study effects of solvent on the formation of these complexes. Binding of aminoglycosides to APH is favored enthalpically and disfavored entropically (*12*, *13*). Typical thermograms and isotherms with fitted curves are shown in Figure S1. The enthalpy of the APH−kanamycin complex was less negative in $D_2O$ relative to $H_2O$. This is commonly observed in protein−carbohydrate interactions (*15*, *25*). Surprisingly, however, formation of the APH−neomycin complex yielded more negative $\Delta H$ in $D_2O$. This prompted us to test a few more members of each group of aminoglycosides. When two other members of the kanamycin group (kanamycin B and tobramycin) and neomycin group (paromomycin and ribostamycin) were used in similar studies, it became clear that binding of kanamycins to APH occurs with more negative enthalpy in $H_2O$ relative to $D_2O$ while the reverse is true for neomycins. $\Delta\Delta H$ ($\Delta H_{H_2O} - \Delta H_{D_2O}$) values are shown in Figure 2.

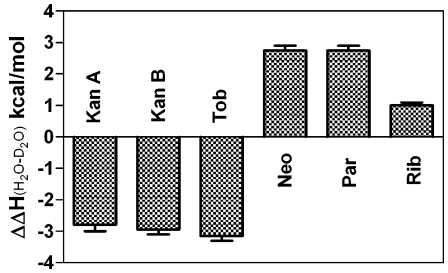



FIGURE 2: $\Delta H(H_2O) - \Delta H(D_2O)$ ($\Delta\Delta H$) values for kanamycins and neomycins. Error bars represent experimental errors.

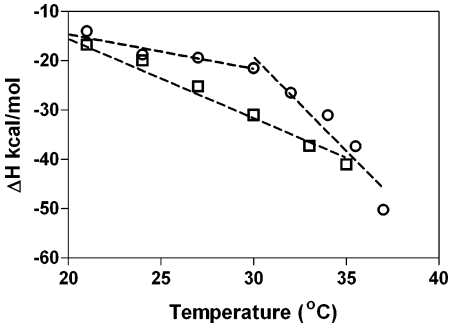



FIGURE 3: Change in $\Delta H$ as a function of temperature for the complexes of APH with neomycin (□) and kanamycin (○). Data are shown with linear regression lines.

It is also known that enthalpies of association can be affected by pH and comparison of enthalpies in $H_2O$ versus $D_2O$ should be made under matched conditions. Since pH-meter readings will be 0.4 units lower in $D_2O$, one may adjust the pH of solutions accordingly. However, p$K_a$s of ionizable groups are also influenced by deuterium which prevents achieving identical conditions in both solutions. For proteins, it was shown that changes in p$K_a$ values for ionizable groups on protein are almost exactly compensated by the difference between hydrogen and deuterium activities (*26*). We have performed our experiments under conditions of pH = pD (no corrections to pH-meter readings), and a few experiments were repeated with a 0.4 unit correction applied to solutions in $D_2O$. The trend described above remained the same only with small quantitative differences between the samples with and without the pH adjustments. Thus, conclusions derived from these experiments were not affected by conditions of sample preparation.

*Determination of the Heat Capacity Change ($\Delta C_p$) for the Formation of APH−Aminoglycoside Complexes*. $\Delta C_p$ is the most relevant thermodynamic parameter related to solvent effects which can be determined from the temperature dependence of $\Delta H$. On the basis of the results described in the previous section, we chose a member from kanamycins (kanamycin) and neomycins (neomycin) to be used for further studies described in the following sections.

Enthalpy changes for the binding of kanamycin and neomycin to APH show strong dependence on temperature, indicating that large negative heat capacity changes accompany binding of these antibiotics to APH (Figure 3). The change in $\Delta H$ for the binding of neomycin to APH showed a linear dependence to temperature and yielded a $\Delta C_p$ of $-1.6$ kcal·mol$^{-1}$·deg$^{-1}$ (Figure 3). Binding of kanamycin to

Table 1: Thermodynamic Data for the Complexes of APH with Aminoglycosides

| | kanamycin−APH | | | neomycin−APH | | |
|---|---|---|---|---|---|---|
| temp (°C) | $\Delta H^a$ (kcal/mol) | $-T\Delta S$ (kcal/mol) | $\Delta G^b$ (kcal/mol) | $\Delta H^a$ (kcal/mol) | $-T\Delta S$ (kcal/mol) | $\Delta G^b$ (kcal/mol) |
| 18 | −13.7 | 6.2 | −7.5 | −14.9 | 6.6 | −8.3 |
| 21 | −14.0 | 6.4 | −7.6 | −16.7 | 8.2 | −8.5 |
| 24 | −18.7 | 10.8 | −7.9 | −19.9 | 11.3 | −8.6 |
| 27 | −19.3 | 11.3 | −8.0 | −25.2 | 16.5 | −8.7 |
| 30 | −21.5 | 13.3 | −8.2 | −31.0 | 22.0 | −9.0 |
| 32 | −26.5 | 18.2 | −8.3 | – | – | – |
| 33 | – | – | – | −37.3 | 27.2 | −10.1 |
| 34 | −31.0 | 22.7 | −8.3 | – | – | – |
| 35 | – | – | – | −41.0 | 30.4 | −10.6 |
| 35.5 | −37.7 | 29.4 | −8.3 | – | – | – |
| 37 | −50.2 | 41.9 | −8.3 | – | – | – |

[a] Errors in $\Delta H$ are between 5% and 15%. [b] Errors in $\Delta G$ are between ±0.1 and ±0.3 kcal·mol$^{-1}$.

APH, on the other hand, showed an apparent break at 30 °C above which $\Delta C_p$ became ∼5-fold more negative. $\Delta C_p$ values for the formation of the APH−kanamycin complex were −0.7 kcal·mol$^{-1}$·deg$^{-1}$ and −3.8 kcal·mol$^{-1}$·deg$^{-1}$ below and above 30 °C respectively. A cubic polynomial function can also be fitted to the data acquired with kanamycin, which yields values similar to those obtained by least-squares fitting of the data below and above 30 °C separately. In all cases, the enthalpic contribution was compensated by the entropic contribution but the compensation was slightly weaker with neomycin yielding a $\Delta\Delta G$ of −2.3 kcal·mol$^{-1}$ between 18 °C and 35 °C. $\Delta\Delta G$ for the kanamycin complex was −0.8 kcal·mol$^{-1}$ for the same temperature range (Table 1).

*NMR Studies of APH−Aminoglycoside Complexes*. Thermodynamic studies yield parameters representing global properties of enzyme−aminoglycoside complexes. In order to understand site-specific implications of these parameters, we used NMR spectroscopy. The $^{15}N-^{1}H$ HSQC (will be referred as HSQC henceforth) spectrum of APH changes dramatically upon binding of aminoglycosides (*27*). The HSQC spectrum of the APH−neomycin complex (Figure 4A) is shown together with the spectrum of the apoenzyme (Figure 4B) acquired under identical conditions with matched samples. While the apoenzyme spectrum is indicative of a structurally flexible molecule and shows significant resonance overlap, the spectrum of the complex shows well-resolved and highly dispersed resonances even for this ∼31 kDa protein (264 amino acids). This suggests that APH is floppy in solution, and it may even be intrinsically unstructured. Addition of aminoglycosides promotes formation of a well-defined structure. This is also consistent with crystallographic studies of the apo and complexed forms of the enzyme where the apoenzyme structure was solved with lower resolution and showed higher temperature factors (*10*, *20*).

HSQC spectra of APH with bound kanamycin and neomycin were acquired to determine the effect of temperature on the backbone amide resonances of APH between 21 °C and 37 °C. As shown in Figure S3, APH−aminoglycoside complexes yield HSQC spectra with highly disperse resonances which allowed temperature-dependent shifts of ∼200 of them to be followed. More than 40 amides showed different temperature dependence between the two complexes. Figure 5 shows plots of chemical shift versus temperature for selected residues in the APH−kanamycin and in APH−neomycin complexes. As illustrated in this figure, some amide protons did not change their chemical shifts as a function of temperature, while some changed in only one of the complexes, and others showed opposite trends. Most notably, ppm versus temperature plots of four backbone amide protons displayed a break at about 30 °C in the APH−kanamycin complex but not in the APH−neomycin complex. These amides belong to residues Leu 88, Ser 91, Cys 98, and Leu143 (resonance assignment of APH−aminoglycoside complexes is to be published upon completion of the assignments, which is currently ∼65% completed. Fortuitously, all four residues that showed the break at 30 °C were among the completed assignments). An expanded region of overlaid HSQC spectra showing the Leu 143 peak as a function of temperature in APH−neomycin and in APH−kanamycin complexes is shown in Figure 6. Spectra in this figure demonstrates that while the proton chemical shift of Leu 143 is different for kanamycin vs neomycin complexes at 21 °C, they merge at 31 °C and continue shifting at similar rates indicating a break for APH−kanamycin complex at ∼30 °C.

## DISCUSSION

Our results show that there are significant differences in the organization of solvent between the complexes of kanamycins and neomycins with APH. Binding studies performed in $H_2O$ and $D_2O$ suggest that $D_2O$ is either destabilizing the unbound state or stabilizing the bound state relative to $H_2O$ with neomycins while the opposite is true for kanamycins. These differences cannot simply be attributed to the differences between the number of hydrogen-bonded groups or trapped solvent molecules in respective enzyme−aminoglycoside complexes because that should yield quantitative differences rather than showing opposite trends observed in $\Delta\Delta H$. In accord with this, Dam et al., (*28*) also found that altered water structure around bound lectins does not necessarily correlate with $\Delta\Delta H$ ($H_2O - D_2O$).

We do not know why there is such a remarkable difference in $\Delta\Delta H$ values of the complexes of kanamycins and neomycins with this enzyme, and we are not aware of another example of a similar behavior in enzymes with multiple substrates. Thermodynamic parameters (including $\Delta H$) of individual complexes of kanamycins and neomycins with the enzyme do not show such group specificity (*13*).

$\Delta\Delta H$ values determined for kanamycins are similar to each other despite significant differences in their $\Delta H$ values. These results suggest that solvent structure is similar in complexes of these aminoglycosides so the differences in their thermodynamic parameters must be due to differential interactions of their functional groups with the enzyme. To this end, the only difference between kanamycin A and kanamycin B is the presence of amino vs hydroxyl at the 2′-position while tobramycin is 3′-deoxykanamycin B (Figure 1). A similar observation in thermodynamic properties was made for the complexes of neomycin and paromomycin (they differ at the 6′-position as amino vs hydroxyl) with APH. However, ribostamycin yielded a smaller but still positive $\Delta\Delta H$ than the two other members of the neomycin group. Ribostamycin lacks ring D, but otherwise it is identical to neomycin.

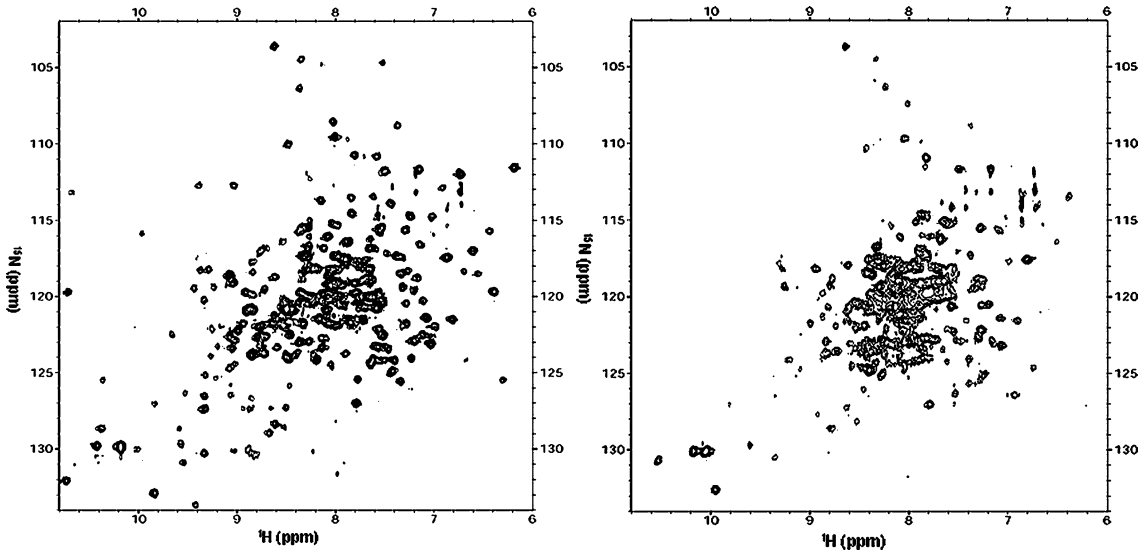



FIGURE 4: $^{15}$N−$^1$H HSQC spectrum of APH−neomycin (left) and apo-APH in (right).

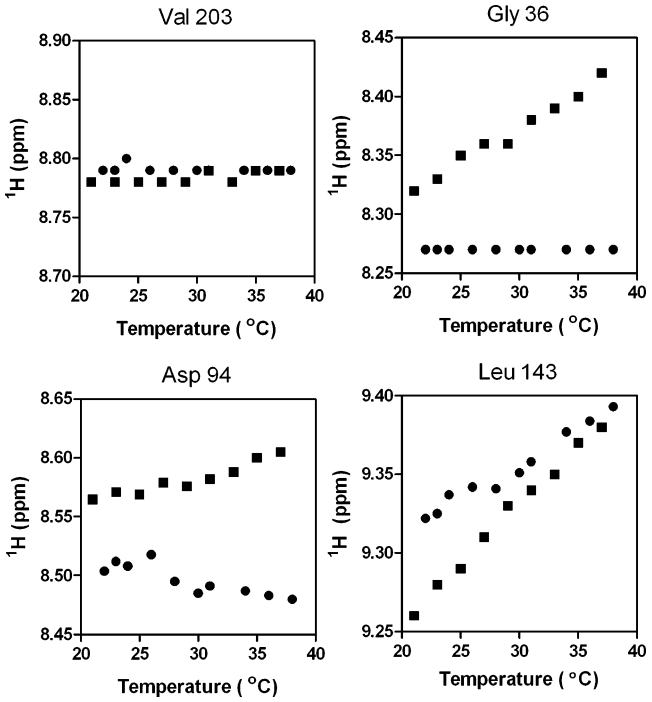



FIGURE 5: Temperature-dependent changes in the proton chemical shifts of several amino acids of APH in complexes with kanamycin (●) and neomycin (■).

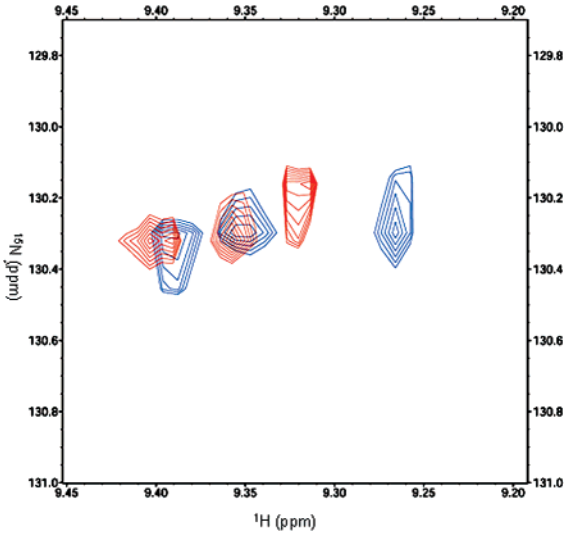



FIGURE 6: Expanded region of $^{15}$N−$^1$H HSQC spectra showing Leu 143 peak as a function of temperature in APH−kanamycin (red, right-to-left 21 °C, 31 °C, and 38 °C, respectively) and in APH−neomycin complex (blue, right-to-left 21 °C, 31 °C, and 37 °C respectively).

Kanamycins also lack ring D. Therefore, it is possible that interactions of the D ring of neomycins with APH cause significant changes in the organization of the solvent in the active site of APH. This is consistent with earlier observations that the 2‴-$NH_2$ (on ring D) of neomycin showed a 1 p$K_a$ unit up shift when bound to APH (*12*), confirming that it is not exposed to solvent in the binary APH−neomycin complex. Since the p$K_a$ of the 6‴-$NH_2$ on the same ring remained unaltered in the same complex, interaction of the 2‴-$NH_2$ with the enzyme must be, in part, responsible for the observed differences of solvent organization in the active site of APH. Interaction of ring D with the enzyme, however, does not completely explain the opposite tendencies observed in $\Delta\Delta H$ values for kanamycins versus neomycins. Ribostamycin, like kanamycins, has three rings; however, it still yields positive, albeit smaller, $\Delta\Delta H$. This indicates that other structural differences between kanamycins and neomycins must also contribute to solvent organization. The main structural difference between these groups is at the 2-deoxystreptamine ring (ring B) which is 4,6- or 4,5-disubstituted in kanamycins and neomycins respectively. Additionally, ring C is a pentose in neomycins and a hexose in kanamycins (Figure 1). In an earlier study Chervenak and Toone (*29*) suggested that differential solute−solute versus solute−solvent hydrogen bonds would produce a more negative $\Delta H$ in $D_2O$. If this is true, then neomycins appear to have different interactions with the enzyme and the solvent.

Another difference between the complexes of neomycins and kanamycins with the enzyme is the orientation of bound substrates with respect to metal−ATP. Neomycins bind to the enzyme in such a manner that rings A and C are much closer to each other allowing inter-ring hydrogen bonds to form (*6*). This conformation allows both the 3′-OH and the 5″-OH to be positioned optimally for a nucleophilic attack on the $\gamma$-phosphate of ATP. Thus either or both hydroxyls can be phosphorylated in neomycins. The bound conforma-

tion of kanamycins does not allow rings A and C to be near each other and only the 3′-OH can be phosphorylated. Strong association of solvent structure and cooperativity of intramolecular hydrogen bonds in sugars are also suggested by MD simulations (*30*). Thus, differences in intramolecular hydrogen bonds of neomycins and kanamycins can alter the solvent structure around bound ligands differentially, which can be one of the main reasons for our observations. Consistent with these, it was also suggested that enthalpy of desolvation depends exquisitely on the arrangement of atoms in the binding site and not simply the number or type (*31*). It remains to be seen whether the same holds for other aminoglycoside-modifying enzymes that are capable of using kanamycins and neomycins as substrates.

Despite the observed opposite trends in $\Delta\Delta H$, the overall $\Delta\Delta G$ varied in a small range because of entropic compensation. Compensation was less pronounced in APH−neomycin complex. We should note, however, that determined $\Delta G$ values may have higher uncertainty because the experiments were optimized for obtaining more reliable $\Delta H$ values (calorimetric $c$ values >20). Thus, the association constants (and derived $\Delta G$) can have larger errors.

Observations described above may have significant implications on how this enzyme accomplishes its biological function. APH is capable of phosphorylating a large number of kanamycins and neomycins, and the apoenzyme appears to be a very flexible molecule in solution (Figure 4B). Thus, the enzyme may achieve binding of structurally different ligands by "adjusting" the solvent structure in the enzyme−ligand complexes, which consequently yields significantly different thermodynamic properties for these complexes.

The parameter most relevant to solvent effects is $\Delta C_p$. Our observations highlight two unusual aspects of aminoglycoside−APH interactions: (a) Change in heat capacity for kanamycin binding to APH yields a break at 30 °C while no such break was observed for binding of neomycin to the same enzyme, (b) $\Delta C_p$ values are unusually more negative than those observed for carbohydrate−protein interactions, which are usually between −0.1 and −0.4 kcal·mol$^{-1}$·deg$^{-1}$.

A break at 30 °C was also observed in a recent study describing binding of netropsin to AATT containing hairpin DNA constructs (*32*). On the basis of crystallographic studies, this behavior was attributed to a water molecule trapped in one of the complexes while it was absent in another, which did not show such a break. In our case, it is not easy to attribute the observed difference between kanamycin and neomycin complexes of APH only to those waters that may be trapped at the ligand−protein interface because NMR studies showed that similar breaks also occurred in the chemical shift changes of several backbone amides as a function of temperature. These residues are in hydrophobic patches distant from the active site (Figure 7). Consistent with these, it was suggested that enthalpic and entropic contributions to a hydrogen bond balances out at about 27 °C (*33*). In addition, in many systems $\Delta H/\Delta S$ values fall on a line with a slope of ∼300 K (*34*). Thus, the break observed in $\Delta C_p$ of the APH−kanamycin complex appears to represent solvent effect on the formation of this complex and indicates that not only a significant contribution of solvent is involved in complex formation, but that the solvent structure is significantly different in complexes of kanamycins relative to those of neomycins. Strong effects of solvent in binding of carbohydrates to protein have been observed (*14*, *15*, *29*, *35*).

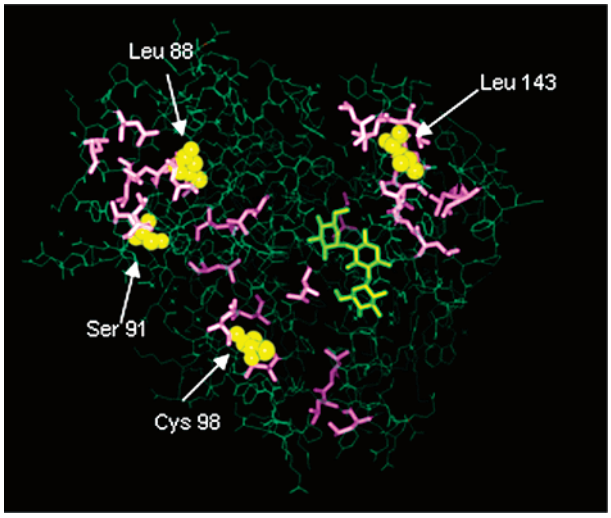



FIGURE 7: Structure of APH−MgADP−kanamycin complex (*10*). Leu 88, Ser 91, Cys 98, and Leu 143 are shown as cpk model while all leucines are shown in pink. Kanamycin is shown in green.

If one assumes that the change in heat capacity is independent of temperature, then one can determine the temperature ($t_H$) where $\Delta H$ would change sign. The formation of APH−neomycin complex would be endothermic below 10 °C ($t_H$ = 10 °C). The low-temperature portion of the APH−kanamycin complex yields $t_H$ value of −1 °C. These results show that formation of both complexes is enthalpically favored under physiological conditions. However, if one were to determine a hypothetical $t_H$ from the high-temperature portion of APH−kanamycin curve, one would get 25 °C. If the break in $\Delta C_p$ of APH−kanamycin complex represents a shift in equilibrium between two conformations of the enzyme, then this result suggests that binding of kanamycin to the dominating conformation above 30 °C would be endothermic below 25 °C. We are not sure what the significance of this is, but it is interesting to note that most of the enzymes that modify aminoglycosides are isolated from bacteria that live in soil where the average temperatures are 23−25 °C while resistance to antibiotics occurs at 37 °C in the human body.

Although large negative heat capacity change has been observed for many systems such as protein−monovalent cation interactions (*36*), drug−protein interactions (*37*), and protein−DNA interactions (*38*−*42*), $\Delta C_p$ for the complexes of kanamycin and neomycin with APH are significantly more negative than those observed for carbohydrate−protein interactions including the most negative value observed for antibody−carbohydrate interaction (*43*). There may be several reasons for that. One of these is the contribution of heat of ionization to the observed thermodynamic parameters. A large contribution of protonation equilibria to $\Delta C_p$ is also observed for DNA−protein interaction (*44*). Neomycin and kanamycin have six and four amino functions, respectively. ITC titrations suggested that p$K_a$s of several amino groups may change upon binding to enzymes that modify aminoglycosides including APH (*13*). There is only one experimentally determined case, where $^{15}$N-enriched neomycin was used to determine p$K_a$s of amine groups in the APH−neomycin complex (*12*). These experiments show that indeed some of

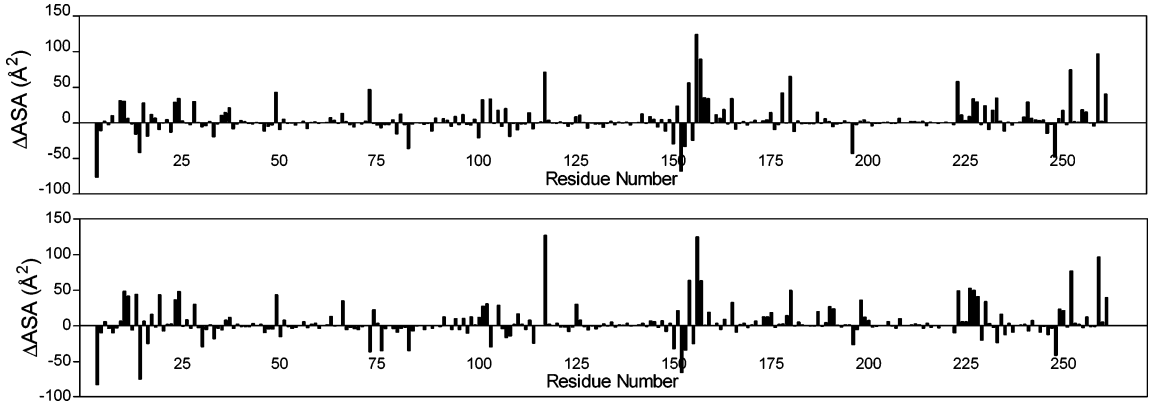



FIGURE 8: Change in the total accessible surface area in the formation of APH−kanamycin (top) and APH−neomycin (bottom) complexes.

the p$K_a$s were shifted up to one p$K$a unit in enzyme-bound neomycin relative to free neomycin. On the basis of the known p$K_a$s, we determined the contribution due to heat of ionization to $\Delta C_p$ as described by Eftink et al. (*45*), which yielded values between −0.12 and −0.14 kcal·mol$^{-1}$·deg$^{-1}$ for the binary enzyme−neomycin complex. By assuming similar increases in p$K_a$ of amino functions in kanamycin, one can estimate a contribution of −0.25 to −0.27 kcal·mol$^{-1}$·deg$^{-1}$ to $\Delta C_p$. Again, all of these values are too small to account completely for the observed decrease in heat capacity. Data presented in Özen et al. (*12*) showed that changes in p$K_a$s of enzymatic sites also occur upon substrate binding, which may contribute to the observed $\Delta C_p$. Thus, the above values that are based only on the ionization of the ligand are obviously underestimated. Contributions from enzymatic sites may be significant.

Negative $\Delta C_p$ values can also be attributed to burial of hydrophobic surfaces, which can explain the observed heat capacity change. Although change in solvent accessible surface area can explain observed changes in heat capacity of protein folding, it usually cannot account for $\Delta C_p$ in many cases such as ligand−macromolecule interactions (*36*, *39*−*42*). To test this, we have determined the changes in accessible surface area (ΔASA) using the crystallographic data. The 'NACCESS' computer program (*46*) was used to determine ASA. The determined ΔASA was 724 Å$^2$ for nonpolar and 1110 Å$^2$ for polar residues for the APH−kanamycin complex. ΔASA values of 806 Å$^2$ and 1031 Å$^2$ were determined for APH−neomycin complex for nonpolar and polar residues, respectively. These values yielded 0.037 kcal·mol$^{-1}$·deg$^{-1}$ and 0.095 kcal·mol$^{-1}$·deg$^{-1}$ for the kanamycin and neomycin complexes, respectively. We also used several methods described in literature (*47*−*49*) to determine upper and lower limits of the contribution of ΔASA to the observed decrease in heat capacity change. This yielded values between −0.037 and −0.133 kcal·mol$^{-1}$·deg$^{-1}$ for both complexes. Thus, even the calculations performed with the method that would yield the most negative $\Delta C_p$ resulted in values that were too small to account for the observed change in heat capacity in complexes of either aminoglycoside with the enzyme.

Figure 8 shows ΔASA for each amino acid in binding of kanamycin to APH. As it can be seen in this plot, significant changes in solvent accessibility were observed for a few regions. Neomycin binding to APH showed changes in solvent accessibility of the same regions as kanamycin binding with only very few differences. This observation indicates that the change in solvent accessible area in both complexes cannot explain the break observed with kanamycin, nor can it account for the large negative values of $\Delta C_p$. Furthermore, it provides no clues to explain the differences observed between the complexes of kanamycin and neomycin with APH.

The heat capacity of water bound to proteins is higher than the bulk water by 2.5 cal·mol$^{-1}$·deg$^{-1}$ (*45*). Binding of aminoglycosides may displace a large number of water molecules from the active site, which may contribute to the observed $\Delta C_p$. Our experiments with several different osmolytes failed to yield consistent results with APH, indicating that there may be specific interactions between the enzyme and some of the osmolytes used. Therefore, data were considered as unreliable. However, our studies with another enzyme that modifies aminoglycoside antibiotics, the aminoglycoside nucleotidyltransferase(2″)-Ia (ANT), were successful with osmolytes. In these studies, binding of tobramycin to ANT displaced about 20 water molecules (*16*). If we assume a similar number of water molecules may be displaced from the active site of APH, then this would yield a $\Delta C_p$ of −0.05 kcal·mol$^{-1}$·deg$^{-1}$, which is, again, a relatively small contribution.

The sum of the above-described contributions still would be too small to account for the large negative $\Delta C_p$ observed for the complexes of both aminoglycosides. Other factors must also be included. In an earlier paper (*50*) Sturtevant suggested that restriction of degrees of freedom of complementary polar, hydrated surfaces contributes to large negative heat capacity. Since these groups are hydrated, corresponding tightening may also occur on vibrational modes of attached water molecules at the interface. He also suggested that this effect may be even more significant for more mobile sugar moieties. Since the aminoglycoside binding site of APH has a large number of polar side chains that interact with amino functions of ligands (Figure S4), this effect is likely to be one of the contributors of the observed decrement to heat capacity.

As indicated earlier, the HSQC spectra of APH are consistent with this enzyme being very flexible in solution and forming a well-defined structure upon aminoglycoside binding. Conformational changes in an enzyme can contribute to the change in heat capacity significantly. For example, in earlier studies, unwinding of a DNA helix or other conformational changes were considered as the likely

contributors to $\Delta C_p$ where ΔASA failed to explain the observed decrease in the change in heat capacity (*38*, *39*). We expect that is the case here and a significant part of the observed $\Delta C_p$ is due to conformational changes in APH. The equilibrium between alternate conformations of the enzyme may shift at about 30 °C, and changes in solvent structure at specific sites may become detectable in some complexes such as the enzyme−kanamycin complex.

An additional contribution to $\Delta C_p$ may also come from conformational changes in ligands. Our earlier studies with several aminoglycosides showed that at least two conformations of APH-bound aminoglycosides are consistent with the observed NOE distance constraints (*7*, *51*, *52*). Furthermore, these conformations can interconvert to each other at 25 °C, and they are different from the conformation of the free aminoglycosides (*51*, *52*). Thus, binding to the enzyme causes conformational changes in ligand as well, which should also contribute to the observed large negative values for $\Delta C_p$ in both complexes studied.

We believe that all of the factors discussed above are in effect in aminoglycoside−enzyme interactions, and their combined effect results in a very large negative $\Delta C_p$. It appears that conformational changes on the enzyme and ligands coupled with the tightening of the vibrational modes of the water molecules at the interface may be the major contributors of the observed large negative $\Delta C_p$ in APH−aminoglycoside complexes. It will be of interest to see whether this applies to other aminoglycoside-modifying enzymes.

In this work, NMR studies of APH−aminoglycoside complexes allowed us to determine specific regions of the enzyme that may give rise to some of the observed effects in the global thermodynamic properties of these complexes. HSQC experiments yielded unexpected results and showed that >40 backbone amide protons displayed a difference between APH−kanamycin and APH−neomycin complexes in their chemical shifts as a function of temperature. Most importantly, four backbone amides paralleled the difference observed in the $\Delta C_p$ values of APH−kanamycin and APH−neomycin complexes. Changes in the proton chemical shifts of Leu 88, Ser 91, Cys 98, and Leu 143 showed a break at ∼30 °C in the APH−kanamycin complex only. This is likely to represent the effect of altered water structure near these residues, which may have affected hydrogen bonds that these protons are involved in. Even the apparently most buried amino acids, Leu 143 and Cys 98, are flanked by hydrophilic and more exposed amino acids. This would allow formation of hydrogen-bonded water structures around these hydrophobic residues, which are shown in Figure 9. Either a conformational change in the enzyme or even a rearrangement of several side chains above the break point may be sufficient to alter the solvent structure in these regions and affect local hydrogen bond networks. It is interesting to note that Leu 143 is at the edge of the segment (147−170) that displays a conformational change upon binding of aminoglycosides (Fong). The C$\gamma$ of Leu 147 is within 4.4 Å of the C$\gamma$ of Leu 143, and both are located in the same hydrophobic patch. Similarly, Cys 98 is also adjacent to a flexible segment (100−112) that shows an altered conformation between the apo- and the ligand-bound form of the enzyme. It is very likely that the solvent structure around the backbone amides of Cys 98 and Leu 143 were altered when conformational changes on the adjacent segments occurred. These changes appear to be different for neomycins and kanamycins. Thus, HSQC experiments may have highlighted specific solvent reorganization sites of APH.

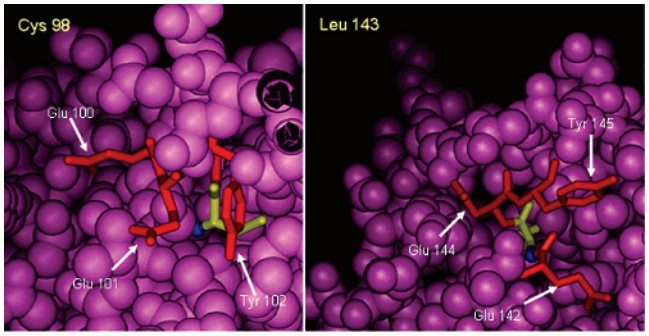



FIGURE 9: Environment of Cys 98 and Leu 143. Left panel: Cys 98 (yellow) and its amide nitrogen (blue) are shown with residues Ser 99, Glu 100, Glu 101, and Tyr 102 (red sticks). Right panel: Leu 143 (yellow with blue nitrogen) is shown with surrounding residues Glu 142, Asp 144, and Tyr 145 (red sticks).

An interesting fact is that most backbone amides that show the break at 30 °C are hydrophobic residues and all are away from the substrate binding site. Furthermore, they are all distributed over three main hydrophobic patches of the protein, which is probably not coincidental. These results also indicate that reorganization of solvent is not limited to the binding interface or its immediate surroundings when a ligand binds to a macromolecule.

## CONCLUSIONS

In this work, we showed that APH is capable of binding its ligands by dramatic alteration in solvent structure in the active site, hence forming enzyme−ligand complexes with significantly different thermodynamic properties. Although some differences between the thermodynamic properties of complexes of kanamycins and neomycins with APH were expected, our findings far exceeded the expected quantitative differences. One remarkable feature was that there was a distinct difference between the complexes of two major groups of aminoglycosides such that one group (neomycins) behaved unusually and yielded more negative $\Delta H$ in $D_2O$ relative to $H_2O$. Part of this was attributed to interaction of the 2‴-$NH_2$ located on ring D of neomycins with the enzyme. However, structural differences in the "core" of kanamycins and neomycins were also a factor in the observed differential effects of $D_2O$ on these complexes. We are not aware of any other enzyme showing similar unusual behavior, but we suspect that other enzymes, capable of binding structurally different ligands, may also show similar properties.

Differences between the complexes of kanamycins and neomycins were also visible in their change of heat capacity. While both showed heat capacities with large negative values, the APH−kanamycin complex revealed a break at 30 °C, above which $\Delta C_p$ became ∼5-fold more negative.

Further manifestation of the observed differences between kanamycin and neomycin complexes of APH was observed in NMR studies. Data showed that, parallel to the break observed in $\Delta C_p$ determinations, there was a break in the temperature-dependent change in the chemical shifts of four backbone amide protons in the APH−kanamycin complex. No such break was visible in any amide protons (or

nitrogens) in the APH−neomycin complex. Most interestingly, these experiments highlighted specific solvent rearrangement sites away from the substrate binding site in the APH−kanamycin complex. Results presented in this paper will hopefully lead further detailed studies of regions of solvent reorganization and their effects on thermodynamic and dynamic properties of enzyme−ligand complexes in general.

## SUPPORTING INFORMATION AVAILABLE

Experimental details. This material is available free of charge via Internet at http://pubs.acs.org.

## REFERENCES


1. Moazed, D., and Noller, H. F. (1987) Interaction of antibiotics with functional sites in 16 S ribosomal RNA, *Nature 327*, 389−394.
2. Umezawa, H. (1974) Biochemical mechanism of resistance to aminoglycosidic antibiotics, *Adv. Carbohydr. Chem. Biochem. 30*, 183−225.
3. Benveniste, R., and Davies, J. (1973) Aminoglycoside Antibiotic-Inactivating Enzymes in Actinomycetes Similar to Those Present in Clinical Isolates of Antibiotic-Resistant Bacteria, *Proc. Natl. Acad. Sci. U.S.A. 70*, 2276−2280.
4. Davies, J. E. (1994) Inactivation of antibiotics and the dissemination of resistance genes, *Science 264*, 375−382.
5. Magnet, S., and Blanchard, J. S. (2005) Molecular insights into aminoglycoside action and resistance, *Chem. Rev. 105*, 477−497.
6. Cox, J. R., McKay, G. A., Wright, G. D., and Serpersu, E. H. (1996) Arrangement of substrates at the active site of an aminoglycoside antibiotic 3′-phosphotransferase as determined by NMR, *J. Am. Chem. Soc. 118*, 1295−1301.
7. Cox, J. R., and Serpersu, E. H. (1997) Biologically important conformations of aminoglycoside antibiotics bound to an aminoglycoside 3′-phosphotransferase as determined by transferred nuclear Overhauser effect spectroscopy, *Biochemistry 36*, 2353−2359.
8. McKay, G. A., Thompson, P. R., and Wright, G. D. (1994) Broad spectrum aminoglycoside phosphotransferase type III from Enterococcus: Overexpression, purification and substrate specificity, *Biochemistry 33*, 6936−6944.
9. McKay, G. A., and Wright, G. D. (1995) Kinetic Mechanism of Aminoglycoside Phosphotransferase Type Iiia - Evidence for a Theorell-Chance Mechanism, *J. Biol. Chem. 270*, 24686−24692.
10. Fong, D. H., and Berghuis, A. M. (2002) Substrate promiscuity of an aminoglycoside antibiotic resistance enzyme via target mimicry, *EMBO J. 21*, 2323−2331.
11. Liu, M., Haddad, J., Azucena, E., Kotra, L. P., Kirzhner, M., and Mobashery, S. (2000) Tethered bisubstrate derivatives as probes for mechanism and as inhibitors of aminoglycoside 3′-phosphotransferases, *J. Org. Chem. 65*, 7422−31.
12. Ozen, C., Malek, J. M., and Serpersu, E. H. (2006) Dissection of aminoglycoside−enzyme interactions: A calorimetric and NMR study of neomycin B binding to the aminoglycoside phosphotransferase(3′)-IIIa, *J. Am. Chem. Soc. 128*, 15248−15254.
13. Ozen, C., and Serpersu, E. H. (2004) Thermodynamics of aminoglycoside binding to aminoglycoside-3′-phosphotransferase IIIa studied by isothermal titration calorimetry, *Biochemistry 43*, 14667−14675.
14. Burkhalter, N. F., Dimick, S. M., and Toone, E. J. (2000) Protein-Carbohydrate interaction: Fundamental considerations, in *Carbohydtrates in Chemistry and Biology* (B. Ernst, G. W. H., and P. Sinay, Ed.) pp 863−914, Wiley-VCH, New York.
15. Dam, T. K., and Brewer, F. C. (2002) Thermodynamic studies of lectin-carbohydrate interactions by isothermal titration calorimetry, *Chem. Rev. 102*, 387−429.
16. Wright, E., and Serpersu, E. H. (2006) Molecular Determinants of Affinity for Aminoglycoside Binding to the Aminoglycoside Nucleotidyltransferase(2″)-Ia, *Biochemistry 45*, 10243−10250.
17. Wright, E., and Serpersu, E. H. (2005) Enzyme-Substrate Interactions with an Antibiotic Resistance Enzyme: Aminoglycoside Nucleotidyltransferase(2″)-Ia Characterized by Kinetic and Thermodynamic Methods, *Biochemistry 44*, 11581−11591.
18. McKay, G. A., Roestamadji, J., Mobashery, S., and Wright, G. D. (1996) Recognition of aminoglycoside antibiotics by enterococcal-staphylococcal aminoglycoside 3′-phosphotransferase type IIIa: Role of substrate amino groups, *Antimicrob. Agents Chemother. 40*, 2648−2650.
19. Kaul, M., Barbieri, C. M., Srinivasan, A. R., and Pilch, D. S. (2007) Molecular Determinants of Antibiotic Recognition and Resistance by Aminoglycoside Phosphotransferase (3′)-IIIa: A Calorimetric and Mutational Analysis, *J. Mol. Biol. 369*, 142−156.
20. Burk, D. L., Hon, W. C., Leung, A. K. -W., and Berghuis, A. M. (2001) Structural analyses of nucleotide binding to an aminoglycoside phosphotransferase, *Biochemistry 40*, 8756−8764.
21. Kay, L. E., Keifer, P., and Saarinen, T. (1992) Pure Absorption Gradient Enhanced Heteronuclear Single Quantum Correlation Spectroscopy with Improved Sensitivity, *J. Am. Chem. Soc. 114*, 10663−10665.
22. Pervushin, K., Riek, R., Wider, G., and Wuthrich, K. (1997) Attenuated T-2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution, *Proc. Natl. Acad. Sci. U.S.A. 94*, 12366−12371.
23. States, D. J., Haberkorn, R. A., and Ruben, D. J. (1982) A Two-Dimensional Nuclear Overhauser Experiment with Pure Absorption Phase in 4 Quadrants, *J. Magn. Reson. 48*, 286−292.
24. Johnson, B. A., and Blevins, R. A. (1994) Nmr View - a Computer-Program for the Visualization and Analysis of Nmr Data, *J. Biomol. NMR 4*, 603−614.
25. Chervenak, M. C., and Toone, E. J. (1995) Calorimetric Analysis of the Binding of Lectins with Overlapping Carbohydrate-Binding Ligand Specificities, *Biochemistry 34*, 7966−7966.
26. Bundi, A., and Wuthrich, K. (1979) Use of Amide H-1-Nmr Titration Shifts for Studies of Polypeptide Conformation, *Biopolymers 18*, 299−311.
27. Welch, K. T., Virga, K. G., Whittemore, N. A., Ozen, C., Wright, E., Brown, C. L., Lee, R. E., and Serpersu, E. H. (2005) Discovery of non-carbohydrate inhibitors of aminoglycoside-modifying enzymes, *Bioorg. Med. Chem. 13*, 6252−6263.
28. Dam, T. K., Oscarson, S., Sacchettini, J. C., and Brewer, C. F. (1998) Differential solvation of "core" trimannoside complexes of the Dioclea grandiflora lectin and concanavalin A detected by primary solvent isotope effects in isothermal titration microcalorimetry, *J. Biol. Chem. 273*, 32826−32832.
29. Chervenak, M. C., and Toone, E. J. (1994) A Direct Measure of the Contribution of Solvent Reorganization to the Enthalpy of Ligand-Binding. Decreased dH in $D_2O$ represents differential interactions with solute and solvent, *J. Am. Chem. Soc. 116*, 10533−10539.
30. Dashnau, J. L., Sharp, K. A., and Vanderkooi, J. M. (2005) Carbohydrate intramolecular hydrogen bonding cooperativity and its effect on water structure, *J. Phys. Chem. B 109*, 24152−24159.
31. Isbister, B. D., Phaedria, M. S. H., and Toone, E. J. (1995) Spatial organization versus total surface area as a predictor of protein hydrophobicity of the concanavalin A binding site, *J. Am. Chem. Soc. 117*, 12877−12878.
32. Freyer, M. W., Buscaglia, R., Hollingsworth, A., Ramos, J., Blynn, M., Pratt, R., Wilson, W. D., and Lewis, E. A. (2007) Break in the heat capacity change at 303 K for complex binding of netropsin to AATT containing hairpin DNA constructs, *Biophys. J. 92*, 2516−2522.
33. Dunitz, J. D. (1995) Win Some, Lose Some - Enthalpy-Entropy Compensation in Weak Intermolecular Interactions, *Chem. Biol. 2*, 709−712.
34. Cooper, A., Johnson, C. M., Lakey, J. H., and Nollmann, M. (2001) heat does not come in different colors: entropy-enthalpy compensation, free energy windows, quantum confinement, pressure perturbation calorimetry, solvation and multiple causes of heat capacity effects in biomolecular interactions, *Biophys. Chem. 93*, 215−230.
35. Daranas, A. H., Shimizu, H., and Homans, S. W. (2004) Thermodynamics of binding of D-galactose and deoxy derivatives thereof to the L-arabinose-binding protein, *J. Am. Chem. Soc. 126*, 11870−11876.
36. Guinto, E. R., and DiCera, E. (1996) Large heat capacity change in a protein−monovalent cation interaction, *Biochemistry 35*, 8800−8804.
37. Connelly, P. R., Thomson, J. A., Fitzgibbon, M. J., and Bruzzese, F. J. (1993) Probing Hydration Contributions to the Thermodynamics of Ligand-Binding by Proteins. Enthalpy and Heat-

Capacity Changes of Tacrolimus and Rapamycin Binding to Fk506 Binding-Protein in $D_2O$ and $H_2O$, *Biochemistry 32*, 5583−5590.
38. Petri, V., Hsieh, M., and Brenowitz, M. (1995) Thermodynamic and Kinetic Characterization of the Binding of the Tata-Binding Protein to the Adenovirus E4 Promoter, *Biochemistry 34*, 9977−9984.
39. Datta, K., Wowor, A. J., Richard, A. J., and LiCata, V. J. (2006) Temperature dependence and thermodynamics of Klenow polymerase binding to primed-template DNA, *Biophys. J. 90*, 1739−1751.
40. Izutani, Y., Kanaori, K., Imoto, T., and Oda, M. (2005) Interaction of gymnemic acid with cyclodextrins analyzed by isothermal titration calorimetry, NMR and dynamic light scattering, *FEBS J. 272*, 6154−6160.
41. Berger, C., Jelesarov, I., and Bosshard, H. R. (1996) Coupled folding and site-specific binding of the GCN4-bZIP transcription factor to the AP-1 and ATF/CREB DNA sites studied by microcalorimetry, *Biochemistry 35*, 14984−14991.
42. Ladbury, J. E., Wright, J. G., Sturtevant, J. M., and Sigler, P. B. (1994) A Thermodynamic Study of the Trp Repressor-Operator Interaction, *J. Mol. Biol. 238*, 669−681.
43. Sigurskjold, B. W., and Bundle, D. R. (1992) Thermodynamics of Oligosaccharide Binding to a Monoclonal-Antibody Specific for a Salmonella O-Antigen Point to Hydrophobic Interactions in the Binding-Site, *J. Biol. Chem. 267*, 8371−8376.
44. Kozlov, A. G., and Lohman, T. M. (2000) Large contributions of coupled protonation equilibria to the observed enthalpy and neat capacity changes for ssDNA binding to Escherichia coli SSB protein, *Proteins*, 8−22.
45. Eftink, M. R., Anusiem, A. C., and Biltonen, R. L. (1983) Enthalpy Entropy Compensation and Heat-Capacity Changes for Protein Ligand Interactions. General Thermodynamic Models and Data for the Binding of Nucleotides to Ribonuclease-A, *Biochemistry 22*, 3884−3896.
46. Hubbard, S. J., and Thornton, J. M. (1993) 'NACCESS' Computer Program, Department of Biochemistry and Molecular Biology, University College, London.
47. Myers, J. K., Pace, C. N., and Scholtz, J. M. (1995) Denaturant M-Values and Heat-Capacity Changes - Relation to Changes in Accessible Surface-Areas of Protein Unfolding, *Protein Sci. 4*, 2138−2148.
48. Spolar, R. S., Livingstone, J. R., and Record, M. T. (1992) Use of Liquid-Hydrocarbon and Amide Transfer Data to Estimate Contributions to Thermodynamic Functions of Protein Folding from the Removal of Nonpolar and Polar Surface from Water, *Biochemistry 31*, 3947−3955.
49. Murphy, K. P., Bhakuni, V., Xie, D., and Freire, E. (1992) Molecular-Basis of Cooperativity in Protein Folding. 3. Structural Identification of Cooperative Folding Units and Folding Intermediates, *J. Mol. Biol. 227*, 293−306.
50. Sturtevant, J. M. (1977) Heat-Capacity and Entropy Changes in Processes Involving Proteins, *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236−2240.
51. Mohler, M. L., Cox, J. R., and Serpersu, E. H. (1998) Aminoglycoside phosphotransferase(3′)-IIIa (APH (3′)-IIIa)-bound conformation of the aminoglycoside lividomycin A characterized by NMR, *Carbohyd. Lett. 3*, 17−24.
52. Cox, J. R., Ekman, D. R., DiGiammarino, E. L., Akal-Strader, A., and Serpersu, E. H. (2000) Aminoglycoside antibiotics bound to aminoglycoside-detoxifying enzymes and RNA adopt similar conformations, *Cell Biochem. Biophys. 33*, 297−308.

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