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Dissection of Aminoglycoside−Enzyme Interactions: A Calorimetric and NMR Study of Neomycin B Binding to the Aminoglycoside Phosphotransferase(3′)-IIIa

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Abstract: In this work, for the first time, we report pK_a values of the amino functions in a target-bound aminoglycoside antibiotic, which permitted dissection of the thermodynamic properties of an enzyme−aminoglycoside complex. Uniformly enriched ^{15}N-neomycin was isolated from cultures of *Streptomyces fradiae* and used to study its binding to the aminoglycoside phosphotransferase(3′)-IIIa (APH) by ^{15}N NMR spectroscopy. ^{15}N NMR studies showed that binding of neomycin to APH causes upshifts of ∼1 pK_a unit for the amines N2′ and N2‴ while N6′ experienced a 0.3 pK_a unit shift. The pK_a of N6‴ remained unaltered, and resonances of N1 and N3 showed significant broadening upon binding to the enzyme. The binding-linked protonation and pH dependence of the association constant (K_b) for the enzyme−aminoglycoside complex was determined by isothermal titration calorimetry. The enthalpy of binding became more favorable (negative) with increasing pH. At high pH, binding-linked protonation was attributable mostly to the amino functions of neomycin; however, at neutral pH, functional groups of the enzyme, possibly remote from the active site, also underwent protonation/deprotonation upon formation of the binary enzyme−neomycin complex. The K_b for the enzyme−neomycin complex showed a complicated dependence on pH, indicating that multiple interactions may affect the affinity of the ligand to the enzyme and altered conditions, such as pH, may favor one or another. This work highlights the importance of determining thermodynamic parameters of aminoglycoside−target interactions under different conditions before making attributions to specific sites and their effects on these global parameters.

Introduction

Aminoglycosides are potent, broad-spectrum antibiotics. They exert their bactericidal effect by targeting 16S rRNA of the 30S ribosomal subunit and interfering with the translational fidelity of protein synthesis.[1−4] The majority of aminoglycosides belong to the 2-deoxystreptamine (2-DOS) structural group, which is composed of a cyclitol ring and amino sugars connected to it by glycosidic bonds. Figure 1 shows the structure of the aminoglycoside neomycin B (will be referred to as neomycin henceforth for simplicity).

The bactericidal effectiveness of aminoglycosides was severely reduced in the recent decades due to the emergence of bacterial strains that are resistant to their action. Among several resistance modes employed by pathogenic bacteria, detoxification of aminoglycoside antibiotics through covalent modification is the most common mechanism.[4,5] A large number of enzymes, produced by these bacteria, can acetylate, nucleotidylate, or phosphorylate the aminoglycoside antibiotics and render them useless. The aminoglycoside phosphotransferase(3′)-IIIa (APH) is the most widespread resistance enzyme in the aminoglycoside phosphotransferase family of modification enzymes. It carries out MgATP-dependent *O*-phosphorylation of the 3′-OH or 5″-OH of aminoglycosides. Due to its wide substrate selectivity

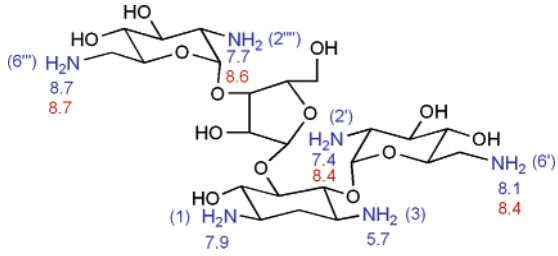

Figure 1. Structure of neomycin B. Amino groups are shown with their numbering indicated in parantheses. The pK_a values of the amino groups in free neomycin[16] and in the neomycin−APH complex (this work) are shown in blue and red, respectively.

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and expression, APH has been the subject of various structural, kinetic, and mechanistic studies.[6−11] However, there are only limited thermodynamic data available for this enzyme and its interactions with substrates.[12,13] Our earlier results indicated that aminoglycoside−APH association was enthalpically favored and entropically disfavored,[13] which is typical for carbohydrate−protein interactions.[14,15] Binding of aminoglycosides to the enzyme was also coupled to protonation of substrates and/or the enzyme. However, detailed analysis of thermodynamic parameters of enzyme−aminoglycoside association was not feasible due to involvement of multiple ionizable groups in binding. In this paper, we present, for the first time, data obtained by direct measurement of the pK_a values of the amino groups of an aminoglycoside, neomycin, in an aminoglycoside−target complex. These data were used to dissect neomycin−enzyme interactions and determine contributions of individual amino groups of the ligand to the global thermodynamic properties of the enzyme−aminoglycoside complex. Our results show that binding of aminoglycosides to the enzyme causes upshifts in the pK_a values of amino groups on the ligand. However, the binding affinity of aminoglycosides to APH shows a complex dependence on pH, and increased positive charge on the aminoglycoside does not always increase the affinity.

Materials and Methods

Aminoglycoside antibiotics and all other general chemicals were obtained from Sigma (St. Louis, MO). D_2O (99.9%) was from Wilmad LabGlass (Buena, NJ), and ^{15}N-enriched (98%) NH_4Cl was from Cambridge Isotope Laboratories (Andover, MA).

Protein Purification. Purification of APH was described previously[8] with the modifications described by Özen and Serpersu.[13] Purified APH was concentrated using Millipore (Billerica, MA) ultrafiltration membranes and stored at −80 °C. Care was taken to ensure that APH remained as a monomeric protein at all times, and the monomeric state of the enzyme was confirmed by HPLC. The reason for taking these precautions is that APH is known to form dimers via two intermolecular disulfide bonds,[8] and as shown below, the aminoglycoside binding to the dimer is significantly different from its binding to the monomeric enzyme. We have shown that the monomeric enzyme binds the aminoglycosides with a 1:1 stoichiometry.[13] However, as shown in Figure 2, our experiments with a largely dimeric enzyme (>90%) demonstrated that one of the monomers has a more than 3 orders of magnitude weakened affinity to aminoglycosides and only tight-binding aminoglycosides, such as neomycin, can populate this low-affinity site. Kanamycin binds half-stoichiometrically under similar conditions. A similar half-stoichiometric binding of aminoglycosides to aminoglycoside acetyltransferase(6′)-Iy (AAC) was also observed with the dimeric enzyme.[17] Therefore, all experiments described in this paper were performed with enzyme preparations that were confirmed to be in the monomeric state.

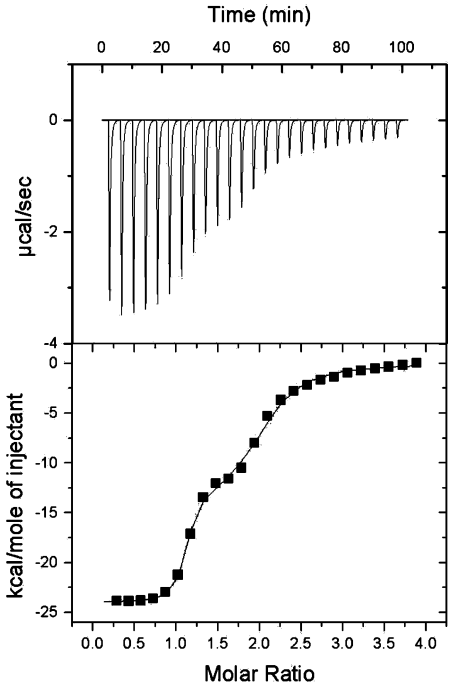

Figure 2. Titration of dimeric APH with neomycin. The thermogram (upper panel) is shown with the fitted data (lower panel), which yields association constants of $1.13 \pm 0.37 \times 10^8$ and $5.9 \pm 0.9 \times 10^5$ M^{-1} for neomycin binding to the first and second binding sites, respectively.

Production and Purification of ^{15}N-Enriched Neomycin B. *Streptomyces fradiae* was obtained from the American Type Culture Collection (Manassas, VA), and the cells were grown in ISP 4 medium supplemented with $^{15}NH_4Cl$ as the sole nitrogen source in a New Brunswick (Edison, NJ) BioFlo 110 fermentor. The cells were grown at 28 °C for 7 days. Following centrifugation, neomycin was isolated from the supernatant by a method similar to those used for the isolation and purification of kanamycin A[18] and ribostamycin;[19] the supernatant was concentrated and loaded into an IRC-50 weak cation exchange column. HCl (1.0 M) was used for elution, and fractions containing neomycin were combined. Following pH adjustment to 7.0, the sample was concentrated to dryness and treated with methanol/acetone precipitation steps. Desiccated powder was dissolved in 10% methanol and loaded into a C18 HPLC column for further purification using 10% methanol as the mobile phase. The product and its purity were verified by NMR spectroscopy (Figure s1 in the Supporting Information) and enzymatic assays.

^{15}N NMR Spectroscopy of Aminoglycosides. ^{15}N-neomycin was diluted from a stock solution to a Shigemi tube (Allison Park, PA) in a total volume of 275 μL at a concentration of 0.5 mM in 90:10 (v/v) H_2O/D_2O. Due to the limited availability of ^{15}N-neomycin and the requirement of a high enzyme-to-neomycin ratio, we initially tried indirect detection by $^{15}N-^{1}H$ heteronuclear single-quantum correlation (HSQC) experiments to achieve higher sensitivity. However, the results were not satisfactory, and therefore, 1D direct detection ^{15}N NMR spectroscopy was used. One-dimensional, ^{1}H-decoupled ^{15}N NMR spectra were recorded as a function of pH for the free and enzyme-bound neomycin at 60.78 MHz on a Varian Inova 600 MHz spectrometer at 25 °C. Although not very high, the S/N ratio was sufficient to detect even crossovers of nitrogen signals in these experiments (Figure 3). When present, APH was ∼0.55 mM to ensure that more than 95% of the neomycin was bound to the enzyme except

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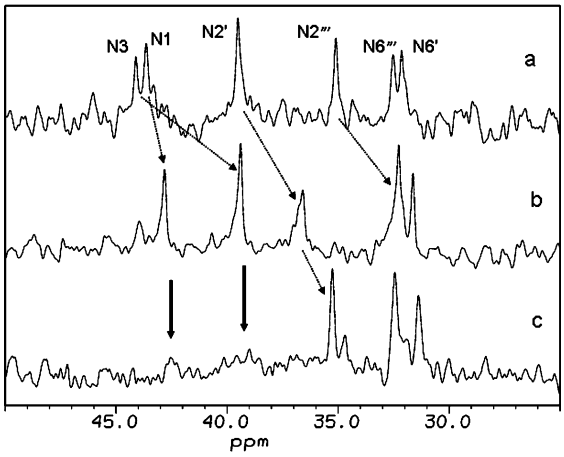

Figure 3. ^{15}N NMR spectrum of 0.5 mM free ^{15}N-neomycin at pH 4.63 (a), at pH 6.80 (b), and at pH 6.73 in the presence of 0.55 mM APH (c). The resonance assignments shown at the top are based on the earlier work by Botto and Coxon.[16]

at pH > 9.0, where the occupancy was ~80. Acquisition parameters included a spectral width of 6000 Hz, a 45° pulse length of 20 μs, and a delay of 0.5 s between scans. A total of 8−16K data points of 20000−80000 FIDs were collected. Before Fourier transformation, 10 Hz line broadening was applied. All ^{15}N chemical shifts are referenced to ^{15}-NH_4Cl. The $^{15}NH_4Cl$ (5% enriched) solution was 0.1 M and was prepared in 90:10 (v/v) H_2O/D_2O. The pK_a values of the amino groups of neomycin were determined by fitting the data, plotted as parts per million vs pH, to a nonlinear regression fit using the GraphPad Prism, version 3.02, for Windows, GraphPad Software (San Diego, CA).

Isothermal Titration Calorimetry (ITC) Experiments. A VP-ITC microcalorimeter from Microcal, Inc. (Northampton, MA) was used to conduct the calorimetry experiments. The experimental temperature was 24 °C. A triple-buffer system composed of 25 mM MES, 25 mM HEPES, and 25 mM BICINE was used in the experiments where the binding affinity of aminoglycosides to the enzyme was determined as a function of pH. A single buffer system of 50 mM ACES, PIPES, TRIS, HEPES, BICINE, or TAPS was used for the determination of proton uptake by enzyme−aminoglycoside complexes at different pH values. In all experiments, the final potassium ion concentration was fixed to 100 mM using KCl. Each series of ITC experiments were performed with the same batch of APH that was extensively dialyzed against the ITC buffer, and ligand solutions were prepared in the final dialysate. As indicated above, freshly added DTT was always present in enzyme solutions to prevent dimerization. A 50 μM enzyme solution is titrated with 0.5 mM neomycin under different conditions. Protein and ligand solutions were degassed under vacuum for 10 min before being loaded into the calorimeter cell and syringe. Titrations consisted of 29 injections programmed as 10 μL per injection and separated by 240 s. The cell stirring speed was 300 rpm. The binding constant (K_b) and enthalpy change (ΔH) were obtained by nonlinear least-squares fitting of experimental data using a single-site binding model of the Origin software package (version 5.0). The *C*-value, a parameter obtained by the multiplication of the association constant (K_b) and the total concentration of ligand binding sites,[20] was within the range of 1−200 in all experiments where reliable determination of the affinity constant is possible. (Abbreviations: MES, 2-morpholinoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; BICINE, *N*,*N*-bis(2-hydroxyethyl) glycine; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol; TAPS, [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid; DTT, dithiothreitol.)

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Results and Discussion

We employed a combination of NMR and ITC to investigate the role of ionizable groups and proton linkage in neomycin binding to APH. ITC was used to determine the net contribution of ionizable groups to the global thermodynamic properties of the binary enzyme−aminoglycoside complex, while NMR spectroscopy was used to identify changes in the pK_a values of amino groups on the enzyme-bound antibiotic neomycin to determine their individual contributions to the thermodynamics of the APH−neomycin complex.

NMR Studies of Free and Enzyme-Bound Neomycin. Studies of aminoglycosides by ^{15}N NMR have been limited to the use of free aminoglycosides because a high concentration is required for detection and ^{15}N NMR studies of target-bound aminoglycosides are simply not feasible. Therefore, to date, the pK_a values of amino functions are determined only for free aminoglycosides.[7,16,21−23] In this work, we report the first determination of pK_a values in a target-bound aminoglycoside antibiotic by using uniformly enriched ^{15}N-neomycin, which was prepared from cultures of *S. fradiae* grown in ^{15}N-enriched media as described in the Materials and Methods.

To determine the effects of binding on each amino group of neomycin, we acquired NMR spectra as a function of pH using ^{15}N-enriched neomycin in the absence and presence of APH (Figure 3). Our ^{15}N NMR spectra matched earlier data acquired with isotopically normal neomycin,[16] and therefore, previously made resonance assignments were applied to our spectra acquired with ^{15}N-neomycin. When present, the enzyme concentration was above the neomycin concentration such that more than 95% of the neomycin was in the binary enzyme−neomycin complex except at very high pH (>9.1, where ~20% of the neomycin was free). The determined pK_a values of the amino groups in free neomycin agreed with the earlier work of Botto and Coxon[16] performed with isotopically normal neomycin in the absence of sulfate (sulfate is present in commercial preparations of aminoglycosides and, if not removed, alters the pK_a values of the amino groups significantly).

Addition of enzyme to the neomycin solution showed that resonances of N1 and N3 were significantly broadened while N2′ was upfield shifted (Figure 3). Broadening of N1 and N3 indicates that exchange rates are at an intermediary stage with these amino groups in the enzyme-bound neomycin, which precluded determination of their pK_a values. These observations are consistent with these three nitrogens being part of a "recognition motif" that adopts the same conformation when bound to enzymes or RNA.[22,24,25] Also, APH shows a strong preference for the aminoglycosides with $-NH_2$ at the 2′-position over those with a $-OH$,[13] which is consistent with the observed upfield shift of this resonance upon binding to the enzyme.

Chemical shifts of the four nitrogen signals representing the 2′-, 2‴-, 6‴-, and 6′-NH_2 groups were followed with varying pH. The change in their chemical shift as a function of pH is shown in Figure 4. There was also a general broadening of these signals between pH values of ~8.7 and 9.1, and no reliable

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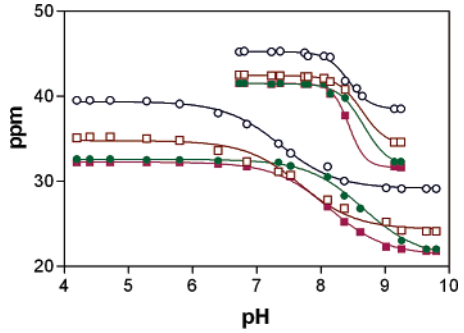

Figure 4. ^{15}N chemical shift titration curves for free (lower curves) and enzyme-bound neomycin (upper curves, which are moved 10 ppm up for clarity): N2′ (○), N2‴ (□), N6′ (■), N6‴ (●).

data points were obtained within this range for the enzyme−neomycin complex. Better defined end points of titration, however, allowed curve fitting to be applied to determine pK_a values. Therefore, the determined pK_a values for these groups should be considered approximate. When there was an overlap, then the titration curves were constructed to conform with the expected shifts for different nitrogens[16] to yield a smooth sigmoidal curve. The pK_a values of free and enzyme-bound neomycin are given in Table 1. The data shown in Figure 4, however, still clearly demonstrate significant shifts in the pK_a values of the enzyme-bound neomycin; thus, the small uncertainty in the pK_a values of the enzyme-bound neomycin does not alter the conclusions of this work. To the best of our knowledge, these data represent the first pK_a determination of a target-bound aminoglycoside antibiotic.

As shown in Figure 4, N2′ and N2″ amino groups show the largest shifts and change their pK_a values by about 1 pK_a unit. 6′-NH_2 shows an about 0.3 unit upshift in its pK_a, and the N6‴ amino group experiences no change in its pK_a. Examination of the crystal structure of the APH−MgADP−neomycin complex[10] (Figure 5) shows that 6‴-NH_2 is exposed to solvent and the nearest carboxyl group (E230) is more than 4.5 Å away. Thus, no change in its pK_a is consistent with the orientation of enzyme-bound neomycin. The N2′ and N2‴ amino groups showed the largest shifts. The N2′ amino is likely to make a hydrogen bond with D190; similarly, D193 is within a H-bond distance of N2‴. Thus, the shifts observed are consistent with the crystal structure of the enzyme−MgADP−neomycin complex.[10] The 6′-amino group, on the other hand, is within 2.5 Å of the carboxyl group of the Phe 264, which is at the C-terminus. E157 is also ∼4.3 Å away from the 6′-amino group, yet the 6′-NH_2 experiences only a shift of ∼0.3 pK_a unit. This is not consistent with the crystal structure, which suggests that either the dynamic properties or the conformation of the binary enzyme−neomycin complex is different around this amino group compared to its environment in the enzyme−MgADP−neomycin complex.

Of the two amino groups whose pK_a values could not be determined, N1 is within 3 Å of E262 and less than 4 Å away from E160. Similarly, N3 is within a 3 Å distance from E157 and ∼4.2 Å from D261. It is very likely that these groups experience significant shifts. Also, their proximity to more than one carboxyl side chain is consistent with their exchange properties. These observations show that at least three amino groups of neomycin experience an upshift in pK_a, which causes further protonation in these groups upon binding to the enzyme. This number does not include N1 and N3, both of which are expected to have a shifted pK_a. On the basis of these structural considerations, one would think that the increased protonation of the amino groups in neomycin should, in principle, increase the affinity of the antibiotic to the enzyme. However, as will be described later, ITC experiments showed that the binding of neomycin to APH is affected by additional interactions, some of which may still dominate at low pH and reduce the affinity of neomycin to APH despite a highly charged state of this antibiotic.

We note that the NMR data acquired with the binary enzyme−neomycin data are evaluated using the structure of the quaternary enzyme−Mg^{2+}−ADP−neomycin complex due to unavailability of a structure for the binary enzyme−aminoglycoside complex. The selection of the binary complex for these studies is, however, still justified. First, when a metal−nucleotide complex is added to APH or the APH−aminoglycoside complex, no or very small changes are observed in the ^{15}N−^{1}H HSQC spectra. Contrary to this, addition of an aminoglycoside to the enzyme or enzyme−metal−nucleotide complex alters both spectra significantly and yields almost identical spectra for both complexes.[28] These observations suggest that the environment of bound neomycin is similar in the binary and the quaternary complexes; thus, the conclusions of this work may largely be applicable to both complexes. These observations are fortuitous, but the main reason for the selection of the binary system is more significant and involves difficulties of data interpretation. ITC experiments described in the following sections are interpreted with the help of NMR data to understand the contributions of the microscopic protonation event to the calorimetric parameters. This requires that the calorimetrically observed parameters should reflect the behavior of the complex of interest only. While the thermodynamics of a binary system can be described by a single equilibrium, a ternary system can be described by six interdependent equilibria,[29,30] and a complete description of a quaternary system such as APH, Mg^{2+}, nucleotide, and aminoglycoside requires more than a dozen equilibria (in reality it is even more complicated than this because there two bound Mg^{2+} ions can be seen in the crystal structures[10]). Thus, at each titration point, the concentrations of various species will change and all dissociations and associations will contribute to the observed heat of reaction. Therefore, it would be practically impossible to determine the effects of pH on the relevant equilibrium alone (binding of neomycin to the enzyme−Mg^{2+}−nucleotide complex), since all equilibria may be affected differentially as the pH is varied. Thus, attribution of the effects of pH or any other variable to the relevant complex (enzyme−metal−ATP−aminoglycoside) can be very difficult.

Calorimetric Studies. Two different calorimetric approaches were used to determine binding-linked protonation/deprotonation and the effect of pH on the formation of the binary enzyme−neomycin complex. The first method involves determination of the observed enthalpy change (ΔH_{obs}) upon ligand (aminoglycoside) binding to the enzyme in buffers with different ionization

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Table 1. pK_a Values of the Amino Groups in Free and Enzyme-Bound Neomycin[a]

	N1	N3	N2′	N6′	N2‴	N6‴
free neomycin	7.9 ± 0.05	5.7 ± 0.06	7.4 ± 0.04	8.1 ± 0.02	7.7 ± 0.07	8.7 ± 0.04
APH−neomycin	ND	ND	8.4 ± 0.03	8.4 ± 0.02	8.6 ± 0.04	8.7 ± 0.1

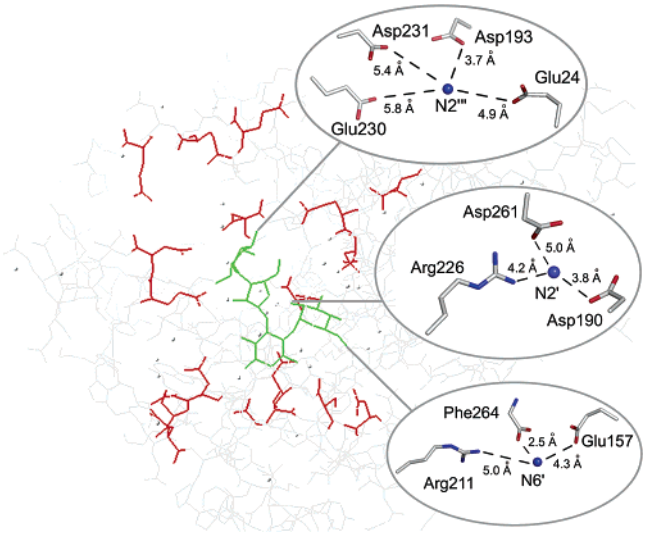

Figure 5. Crystal structure of APH−MgADP−neomycin.[10] Bound neomycin (green) is surrounded by a large number of acidic residues (red). The immediate environment of the three amino groups that show an upshifted pK_a are shown in expanded views. This figure was created by using Pymol[26] and DeepView[27] software.

enthalpies (ΔH_{ion}) to derive the intrinsic enthalpy change (ΔH_{int}) and the net number of binding-coupled proton exchanges (Δn). In the presence of binding-linked protonation, the observed enthalpy (ΔH_{obsd}) includes contributions from various sources according to the equation

$$\Delta H_{obsd} = \Delta H_{int} + \Delta n[\alpha\Delta H_{ion} + (1 - \alpha)\Delta H_{enz}] + \Delta H_{bind}$$ [31]

in which ΔH_{int} is the intrinsic enthalpy of binding, Δn represents the net proton transfer, ΔH_{obsd} denotes the observed binding enthalpy of complex formation in a buffer, ΔH_{ion} describes the heat of ionization of the buffer, the term $\Delta n[\alpha\Delta H_{ion} + (1 - \alpha)\Delta H_{enz}]$ represents the heat of ionization of groups from the ionization of the buffer and the protein to maintain the pH, where α represents the fraction of protonation contributed by the buffer,[31] and ΔH_{bind} represents the heat of binding of the buffer to the enzyme. In the presence of high salt (i.e., 100 mM KCl), ΔH_{bind} is assumed to be zero and the contribution from the ionization of amino acids remains the same at a given pH. Thus, by performing experiments in buffers with different heats of ionization, one can easily determine ΔH_{int} and Δn. However, note that ΔH_{int} still includes the heat of ionization of groups contributing to Δn (i.e., $\Delta H_{int} = (\Delta H_{int} + \Delta H_{functional\ groups}\Delta n)$ which would represent the true ΔH_{int} only when $\Delta n = 0$). For the buffers used in this work, a net proton uptake by the enzyme−ligand complex yields a positive Δn.

Binding-Linked Protonation. Binding-linked protonation was observed in the formation of the binary APH−neomycin complex. Values of ΔH_{int} and Δn showed a strong dependence on pH as shown in Table 2. An interesting observation was made that there appeared to be no net binding-linked protonation or a small deprotonation occurs at pH 6.7 ($\Delta n \approx 0$). This is usually taken as an indication that the determined ΔH_{int} under these conditions may be used as the true ΔH_{int}, which is free of contributions of ΔH_{ion} of the ligand and/or enzyme groups. This is somewhat surprising because one would expect the pK_a of N3 to upshift upon binding to the enzyme due to its proximity to the carboxyl groups (Figure 5). This would alone contribute to the observed Δn by ~+0.4 at this pH. In addition, shifts observed in the pK_a values of N2′ and N2‴ cause a contribution of an additional ~0.24 from these groups, making the overall expected contribution to the observed Δn to be >0.6. Thus, it is likely that deprotonation of another group may compensate for the combined protonation of N3, N2′, and N2‴, yielding an overall $\Delta n \leq 0$. Analysis of the data in Table 2, as described below, lends further support to the suggestion that other protonation/deprotonation reactions also accompany the formation of the enzyme−neomycin complex.

Table 2. pH-Dependent Intrinsic Enthalpy Change (ΔH_{int}) and Net Number of Binding-Coupled Proton Transfers (Δn) for the Neomycin−APH Complex

pH	buffer	ΔH_{ion}[a] (kcal/mol)	ΔH_{obsd}[b] (kcal/mol)	ΔH_{int} (kcal/mol)	Δn
6.7	ACES	7.5	−16.8 ± 1.7	−15.7 ± 2.0	−0.1 ± 0.0
6.7	PIPES	2.7	−16.1 ± 1.6		
7.6	TRIS	11.4	−9.3 ± 0.9	−26.5 ± 3.4	1.5 ± 0.3
7.6	HEPES	5.0	−18.9 ± 1.9		
8.5	BICINE	6.5	−21.9 ± 2.2	−38.0 ± 4.9	2.5 ± 0.4
8.5	TAPS	9.0	−13.3 ± 1.3		

[a] Taken from Fukada and Takahashi, 1998. [b] Determined at 297 K. Uncertainties reflect combined fitting errors and the standard error of mean for the replicates.

If one assumes that ΔH_{int} determined at pH 6.7 is the true intrinsic enthalpy, then one can estimate ΔH_{ion} of groups involved in protonation, which may then yield clues to the nature of these groups. The heat of formation (ΔH_{int}) of the binary APH−neomycin complex becomes more negative with increasing pH. When ΔH_{int}, determined at the regime where $\Delta n = 0$, is subtracted from values obtained at regimes where $\Delta n \neq 0$ and the result divided by Δn, the contribution due to ΔH_{ion} of group(s) involved in binding-linked protonation (groups with a shifted pK_a) may be revealed. Such a calculation yields ΔH_{ion} of −7.2 and −8.9 kcal/mol for the formation of the APH−neomycin complex at pH 7.6 and 8.5, respectively. These values are somewhat lower than ΔH_{ion} of the amino groups, which is about 9−10 kcal/mol.[32] Assuming that the contribution of other factors such as cooperativity of hydrogen bonds to ΔH_{int} remains unaltered, these data suggest that several functional groups may be undergoing protonation/deprotonation reactions. Therefore, the observed ΔH_{ion} represents an "average" value and precludes identification of the types of functional groups with altered pK_a values. These data also show that the value observed for ΔH_{ion} at higher pH contains more contributions from the amino groups since its value is closer to ΔH_{ion} of the amino groups.[32] This

(31) Christensen, T.; Svensson, B.; Sigurskjold, B.W. *Biochemistry* **1999**, *38*, (19), 6300−6310.

(32) Freire, E. *Proc.Natl. Acad. Sci. U.S.A.* **1999**, *96*, 10118−10122.

appears to be a trend in aminoglycoside−enzyme interactions. Even a larger increase in ΔH_{ion} (from −6.4 to −10.8 kcal/mol) was observed with increasing pH in the aminoglycoside nucleotidyltransferase(2″)-Ia−neomycin complex (Wright and Serpersu, unpublished data), which suggests that, at high pH, protonation of the amino groups is likely to be the major contributor to ΔH_{ion} in that complex as well.

We also attempted to dissect contributions of individual amino groups of neomycin to the observed thermodynamic parameters. A simple calculation shows that the protonation of the N2′, N2‴, and N6′ amino functions should contribute to the observed Δn by about 0.97 and 1.0 at pH 7.6 and 8.5, respectively. If the observed Δn were solely due to the protonation of neomycin, then this would leave N3 and N1 as the only amino groups to make up the rest of the observed Δn values. However, no combination of pK_a values can be assigned to these groups that would add up to Δn to match the observed values at both pH values. This, again, strongly suggests that protonation/deprotonation of other groups must contribute to the observed thermodynamic parameters. The active site of APH has only Asp, Glu, and Arg side chains as functional groups (Figure 5), none of which are normally expected to be involved in protonation/deprotonation in the pH regime studied. The likely functional group to contribute in this pH regime may be the imidazole side chain. However, the nearest histidine residue to any part of neomycin is ∼9 Å away and is shielded from neomycin by other residues. Thus, these observations also suggest propagation of effects to remote sites upon binding of aminoglycoside to the enzyme. Such remote effects have been detected with other proteins.[33]

The combined contribution of N2′, N2‴, and N6′ would be ∼10 kcal/mol at pH 7.6. Since they also account for a contribution of ∼1 to Δn, this would leave only 0.8 kcal/mol for an additional Δn of ∼0.5, which is too small for any heat of ionization reaction except for carboxyl groups. This would imply that either there is no change in the pK_a values of N1 and N3, which is very unlikely, or compensatory ionization by other groups contributes to the observed enthalpy. Furthermore, the same calculation for the data obtained at pH 8.5 yields ∼12.3 kcal/mol for the additional Δn of ∼1.5, leaving ∼8.2 kcal/mol, a value much closer to the heat of ionization of the amino groups. In this case, it is possible that, at pH 8.5, almost all of the heat of ionization can be attributed to the protonation of five amino groups in neomycin. Thus, experiments performed at two different pH values suggest that all protonation may occur on the ligand at high pH; however, at pH 7.6, other functional groups also undergo protonation/deprotonation reactions.

Enzyme−Aminoglycoside Association as a Function of pH. The second calorimetric method involves determination of K_b for the formation of binary enzyme−aminoglycoside complexes as a function of pH. The effect of the pH on K_b for the formation of the APH−neomycin complex was investigated by ITC. A pH range of 6.8−9.1 was used in a triple-buffer system.

The dramatic effect of the pH on K_b is shown in Figure 6 in representative ITC titrations. The affinity of neomycin to the enzyme was reduced at high pH. However, K_b does not necessarily correlate with the fractional charge on the aminoglycosides. As shown in Figure 7, the affinity (K_b) of neomycin to the enzyme was increased first with increasing pH up to pH 8.1, showed a plateau between pH 8.1 and 8.4, and then decreased sharply above pH 8.4. The difference between the highest and lowest K_b is more than 60-fold in the APH−neomycin complex. The increase in affinity appears to correlate with Δn and inversely correlate with the total fraction of $-NH_3^+$ ions in neomycin in the pH range of 6.8−8.4. The sum ($\Delta n + [-NH_3^+]$), however, remains essentially unchanged between pH 6.8 and pH 8.4, and yet K_b increases by a factor of ∼6.5-fold within the same pH range. An increase in affinity with increasing pH suggests that deprotonation of a group or more is contributing favor-

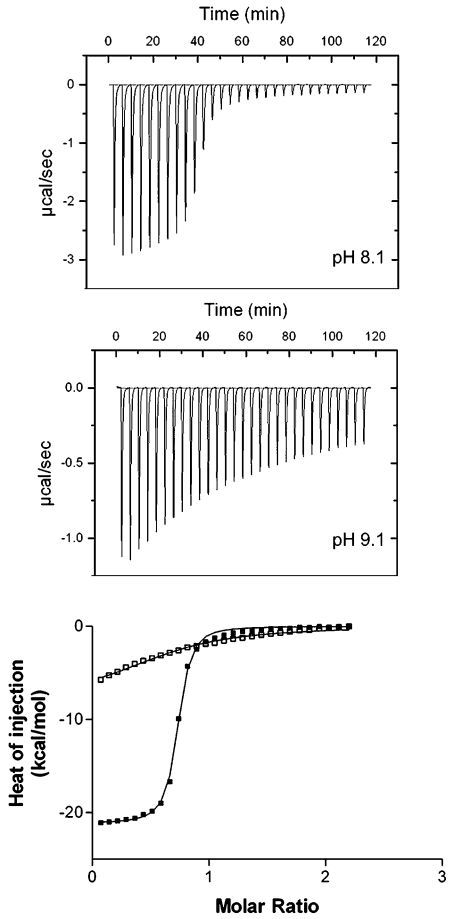

Figure 6. Titration isotherms of neomycin to APH at pH 8.1 (■) and pH 9.1 (□). Raw data (thermal power) are given in the two upper panels. The bottom panels show the heat of injection, which is obtained by integration of the thermal power, plotted against the molar ratio of ligand to enzyme. Nonlinear curve fitting of the binding data is shown with a solid line. The observed binding constants (K_b) obtained from the fits of these and other pH experiments are given in Table 2.

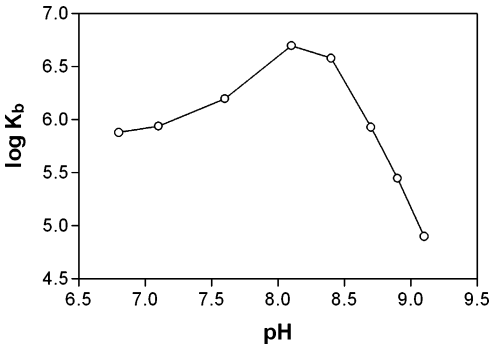

Figure 7. Association constant (K_b) for the binary APH−neomycin complex as a function of pH.

(33) McKay, G. A.; Roestamadji, J.; Mobashery, S.; Wright, G. D. *Antimicrob.Agents Chemother.* **1996**, *40* (11), 2648−2650.

ably to the binding of neomycin to APH. This deprotonation is most likely to be from a functional group(s) in the enzyme.

The increase in K_b of the APH−neomycin complex as the pH is raised from 6.8 to 8.4 is not easy to explain. Using the determined pK_a values of four of the six amino groups in enzyme-bound neomycin, one can now determine the protonation state of the N2′, N2‴, N6′, and N6″ amino groups. At pH 6.8, these groups are >95% protonated. Their protonation is reduced to ∼70% at pH 8.1. Thus, the net positive charge on neomycin experiences a decrease in this pH range, yet it has an increased affinity to the enzyme. It is not clear how the decrease in charge would increase the affinity of neomycin to APH, because almost all amino groups of enzyme-bound neomycin are near carboxyl functions in the active site. The N1 amino and the 2′-amino are the only groups that are near a positively charged enzyme functional group (R226). Early kinetic studies showed that an amino group at N1 and an amino or hydroxyl at the 2′ position is needed for a lower K_m.[34] Binding studies showed that aminoglycosides with an amino function at the 2′ position bind to APH and other aminoglycoside-modifying enzymes tighter than those with a hydroxy at this position and show significantly different thermodynamic properties.[13,35] There are no enzyme functional groups that are good candidates in the active site of APH to undergo protonation/deprotonation in this pH range. Also, the only basic side chain belongs to R226, which, normally, has a too high pK_a to be affected in the pH range studied for binding. This leaves us with an option that either deprotonation of an enzyme side chain remote from the active site is increasing K_b via conformational changes in the enzyme, or as shown in Figure 5, the N2′ amino group of neomycin, despite its higher shifted pK_a, may still contribute favorably upon deprotonation because it is the closest amino group to R226 (∼4.2 Å away). However, it is also near two carboxyl functions, D190 and D261. Thus, the upshift in its pK_a and its contribution to K_b may be determined by the interplay of two opposing effects; while the positively charged N2′ amino interacts favorably with carboxyls, its interaction with R226, on the other hand, may oppose this. In this pH range, the charge on the N2′ amino group is reduced by 30% at pH 8.1 starting from a fully charged (>98%) state at pH 6.8. Thus, as the pH is raised, the unfavorable interaction between this group and R226 will be reduced. Another amino group that may also contribute to this is the N1 amino group, which is ∼4.4 Å away from R226. This is, however, less likely because the pK_a of N1 is already 7.9 in free neomycin and it is likely to be upshifted significantly upon binding because it is near two carboxyl groups. If this is the case, then its bound pK_a may simply be too high to cause a significant deprotonation of N1 in the pH range of 6.8−8.1. In light of this, the observed effect of the pH on K_b may still reflect, in part, deprotonation of the 2′-amino group in neomycin.

(34) Wright, E.;Serpersu, E. H. *Biochemistry* **2005**, *44*, 11581−11591.
(35) Uncertainities represent curve fitting errors.

Conclusions

In this work, we determined the pK_a values of amino groups for an aminoglycoside bound to one of its target molecules, an enzyme causing resistance to these antibiotics. The data permitted us to determine contributions of selected amino groups of the ligand to the global thermodynamic properties of the enzyme−ligand complex. Studies described in this paper show that binding of aminoglycoside antibiotics to APH involves a complex pattern of protonation and deprotonation of substrate amino groups and functional groups of the enzyme. Contributions from different groups at different pH values further complicate data analysis. These findings highlight problems associated with attribution of thermodynamic parameters to specific sites even in a simple binary enzyme−ligand complex and underline the importance of the determination of thermodynamic parameters of a system under different conditions before attributing the observed changes in global thermodynamic parameters to specific residues/sites. These studies also showed that, along with structural studies, studies to determine dynamic and thermodynamic properties of enzyme−aminoglycoside complexes are necessary to understand the factors contributing to the formation of enzyme−aminoglycoside complexes.

Overall, results described in this paper showed that the thermodynamics of the binary enzyme−aminoglycoside complexes are strongly dependent on environmental factors such as the pH and its effect on the ionizable groups of aminoglycosides. Formation of the binary aminoglycoside−APH complex is coupled to a net protonation of several amino groups on the ligand. However, global parameters such as Δn, determined by thermodynamic studies, can sometimes be misleading. In our case, a net protonation was indicated by the positive sign of Δn upon binding of neomycin to APH, but other studies clearly showed that binding was also accompanied by deprotonation of other groups, which was masked in the global parameter Δn. In conclusion, a combination of NMR and calorimetric studies permitted dissection of the thermodynamic properties of an enzyme−aminoglycoside complex, and the contributions of individual sites on the ligand to the formation of the enzyme−ligand complex were determined.

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Supporting Information Available: ^{1}H NMR spectrum of the cell culture medium, Figure S1. This material is available free of charge via the Internet at http://pubs.acs.org.

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