Thermodynamics of Aminoglycoside and Acyl-Coenzyme A Binding to the *Salmonella enterica* AAC(6′)-Iy Aminoglycoside *N*-Acetyltransferase†

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ABSTRACT: Kinetic and mechanistic studies on the chromosomally encoded aminoglycoside 6′-*N*-acetyltransferase, AAC(6′)-Iy, of *Salmonella enterica* that confers resistance toward aminoglycosides have been previously reported [Magnet et al. (2001) *Biochemistry 40*, 3700−3709]. In the present study, equilibrium binding and the thermodynamic parameters of binding of aminoglycosides and acyl-coenzyme A derivatives to AAC(6′)-Iy and of two mutants, C109A and the C109A/C70A double mutant, have been studied using fluorescence spectroscopy and isothermal titration calorimetry (ITC). Association constants for different aminoglycosides varied greatly (4×10^4−150×10^4) while the association constants of several acyl-coenzyme A derivatives were similar (3.2×10^4−4.5×10^4). The association constants and van't Hoff enthalpy changes derived from intrinsic protein fluorescence changes were in agreement with independently measured values from isothermal titration calorimetry studies. Binding of both aminoglycosides and acyl-coenzyme A derivatives is strongly enthalpically driven and revealed opposing negative entropy changes, resulting in enthalpy−entropy compensation. The acetyltransferase exhibited a temperature-dependent binding of tobramycin with a negative heat capacity value of 410 cal mol^{-1} K^{-1}. Isothermal titration studies of acetyl-coenzyme A and tobramycin binding to mutant forms of the enzyme indicated that completely conserved C109 does not play any direct role in the binding of either of the substrates, while C70 is directly involved in aminoglycoside binding. These results are discussed and compared with previous steady-state kinetic studies of the enzyme.

Aminoglycoside antibiotics are clinically used in the treatment of severe bacterial infections, and their primary mechanism of action is the inhibition of bacterial protein synthesis by binding to the small ribosomal subunit (*1*, *2*). Bacteria have developed a variety of resistance mechanisms toward aminoglycosides, including (i) single base alterations of ribosomal RNA and single amino acid substitutions in small ribosomal subunit proteins (*3*, *4*), (ii) decreased uptake and/or accumulation of the drug in bacteria due to changes in the permeability of the outer membrane or active efflux (*5*−*7*), and most commonly (iii) enzymatic modification of the drug (*8*−*12*). Three different types of enzymatic modification of the drug have been demonstrated and include O-phosphorylation, O-nucleotidylation, and N-acetylation. Aminoglycoside *N*-acetyltransferases (AAC)[1] use acetyl-coenzyme A (acetyl-CoA) as the acetyl group donor and are grouped into four classes, AAC(1), AAC(2′), AAC(3), and AAC(6′), on the basis of the site of their regioselective acetylation of the aminoglycoside. Kinetic and mutagenic characterization of the chromosomally encoded *Salmonella enterica* aminoglycoside *N*-acetyltransferase AAC(6′)-Iy, which confers resistance toward a number of aminoglycoside antibiotics, has been reported (*13*). The AAC(6′)-Iy is a homodimer of 17 kDa monomers, and kinetic studies supported a random sequential mechanism for the enzyme. Kinetic studies on two mutant forms of AAC(6′)-Iy, C109A and C109A/C70A, indicated the possible involvement of these cysteine residues, in particular C70, in substrate binding (*13*).

Thermodynamic studies provide valuable information regarding the molecular basis of ligand−receptor interactions and are particularly important in determining the driving forces for the binding process, including contributions from hydrogen-bonding, hydrophobic, and van der Waals interactions. In the present study, we report the equilibrium binding and the directly determined thermodynamic parameters for different aminoglycosides and acyl-coenzyme A derivatives to AAC(6′)-Iy and two mutants, C109A and the C109A/C70A double mutant, using fluorescence spectroscopy and isothermal titration calorimetry. The equilibrium and thermodynamic data are discussed in terms of aminoglycoside structures, their kinetic behavior, synergistic binding of substrates, and comparisons of the energetics described for other carbohydrate-binding proteins.

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[1] Abbreviations: ITC, isothermal titration calorimetry; AAC, aminoglycoside *N*-acetyltransferase; CoA, coenzyme A; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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EXPERIMENTAL PROCEDURES

Materials. All chemicals, coenzyme A derivatives, and aminoglycoside antibiotics were purchased from Sigma-Aldrich Chemical Co. *Escherichia coli* strain BL21(DE3) was obtained from Stratagene and Ni-NTA His.Bind resin was from Novagen.

Expression and Purification of the Proteins. Wild-type AAC(6′)-Iy and mutants were expressed and purified as described previously (*13*).

Preparation of 6′-Acetyltobramycin. Acetyltobramycin was prepared enzymatically from tobramycin using AAC(6′)-Iy and excess acetyl-CoA. The reaction mixture was then loaded onto a Mono Q column to remove coenzyme A, unused acetyl-CoA, and the enzyme. Unbound acetyltobramycin was concentrated and desalted on a 1 × 120 cm Sephadex G-10 column to remove buffer salts and freeze-dried. The product was found to be free of free tobramycin as electrospray ionization mass spectra of the product did not show any peak corresponding to tobramycin.

Protein Estimation. Protein concentrations were estimated by the Bio-Rad protein assay method using bovine serum albumin as a standard.

Fluorescence Measurements. Fluorescence measurements were performed on a FluoroMax-3 spectrofluorometer, using an excitation and emission slit width of 2−2.5 nm. An excitation wavelength of 295 nm was used, and emission was monitored at 348 nm (λ_{max} of the enzymes). The fluorescence quenching measurements were made by titrating 2 mL of a 2.5 μM enzyme solution in 50 mM Tris buffer, pH 7.5, containing 50 mM NaCl, 50 μM DTT, 100 μM EDTA, and 0.02% (v/v) Tween-80 with aminoglycoside and coenzyme A solutions (1−100 μM, 2−10 μL aliquots), followed by monitoring the change in fluorescence at 348 nm. The fluorescence of the buffer and ligand solution was also measured at appropriate wavelength and used to correct the observed fluorescence. Corrections for the volume change upon ligand addition were ignored, as the volume change at the highest ligand concentration was less than 1%. The temperature of the enzyme and ligand solutions was maintained at ±0.1 °C of the specified temperature by using a constant temperature water bath. The relative fluorescence intensities of wild-type and mutant AAC(6′)-Iy, saturated with ligand (F_∞), were obtained from the experimental data by plotting $1/F_o\Delta F$ (in case of decrease in fluorescence intensity) and $F_o/F_o\Delta F$ (in case of increase in fluorescence intensity) versus 1/[L] and extrapolating to the *y*-axis, where F_o is the fluorescence intensity of the enzyme alone and F is the fluorescence intensity of the enzyme at a ligand concentration [L]. $\log(F_o\Delta F/F\Delta F_\infty)$ was plotted against log [L], and the association constant, K_a, was determined from the plot by assuming the relation that the pK_a of the complex equals the value of [L] when $\log(F_o\Delta F/F\Delta F_\infty) = 0$ (*14*). Free energies of association were determined by the equation

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

where T is the absolute temperature and $R = 1.98$ cal mol^{-1} K^{-1}. The enthalpy change (ΔH) was calculated by a van't Hoff analysis from the temperature dependence of K_a using the equation

$$\ln K_a = (-\Delta H/RT) + \Delta S/R \quad (2)$$

The slope of the plot of ln K_a versus $1/T$ equals $-\Delta H/R$, from which the enthalpy change was calculated. The entropy change was then obtained from the equation

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

Isothermal Titration Calorimetry. Calorimetric titrations were performed using a MCS microcalorimeter from Microcal, Inc. (Northampton, MA). All measurements were carried out in 50 mM Tris buffer, pH 7.5, containing 50 mM NaCl, 0.1% 2-mercaptoethanol (v/v), and 0.01% Tween-80 (w/v). Enzyme preparations were dialyzed extensively against the above buffer, and ligand solutions were prepared in the final dialysate. In individual titrations, injections of 3 μL of the ligand solution were made via the computer-controlled microsyringe, at an interval of 4 min, into the enzyme solution (cell volume = 1.34 mL), while being stirred at 300 rpm, at the specified temperature. The enzyme and ligand concentrations, in these experiments, were 85−135 μM and 5−8 mM, respectively. When lividomycin A was titrated in the presence of a saturating concentration of acetyl-CoA, the enzyme solution was preincubated with 1 mM acetyl-CoA, and the same concentration of acetyl-CoA was included in the ligand solution. The instrument was calibrated using the calibration kit supplied by the manufacturer. The quantity $c = K_aM_t(0)$, where $M_t(0)$ is the initial macromolecule concentration, is of importance in titration microcalorimetry (*15*). All experiments were performed with c values in the range of 2−100 in the present study. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (the binding enthalpy change in kcal mol^{-1}), K_a (the binding constant in M^{-1}), and n (the number of binding sites per monomer) as adjustable parameters. The thermodynamic parameters ΔG and ΔS were calculated from eqs 1 and 3. The change in heat capacity of binding (ΔC_p) was determined using the equation

$$\Delta C_p = (\Delta H_{T2} - \Delta H_{T1})/(T_2 - T_1) \quad (4)$$

RESULTS

Fluorescence Titrations. The structures of aminoglycosides used in this study are shown in Figure 1. The addition of aminoglycosides to AAC(6′)-Iy resulted in the enhancement of intrinsic protein fluorescence by 15−60%, except in the case of amikacin (a semisynthetic, N^1-4-amino-2-hydroxybutyryl derivative of kanamycin A) where a decrease of 14% was observed. Titration with butirosin (N^1-4-amino-2-hydroxybutyryl ribostamycin) or netilmicin (N^1-ethyl sisomicin) resulted in only a 2−3% increase in fluorescence, resulting in unreliable data. Neither the single nor double cysteine mutants of AAC(6′)-Iy exhibited any fluorescence changes upon addition of aminoglycosides. The titration of AAC(6′)-Iy and its mutant forms with coenzyme A derivatives resulted in the quenching of intrinsic protein fluorescence with a maximum quenching of 14−58% for different acyl-coenzyme A derivatives. The maximum percentage changes in fluorescence intensity upon titration with acyl-CoA derivatives were identical for wild-type AAC(6′)-Iy and mutant forms. The change in fluorescence upon titration with aminoglycosides and coenzyme A derivatives was associated with a very small blue shift in the fluorescence maxima from 348 to 346 nm (Figure 2). The association constants

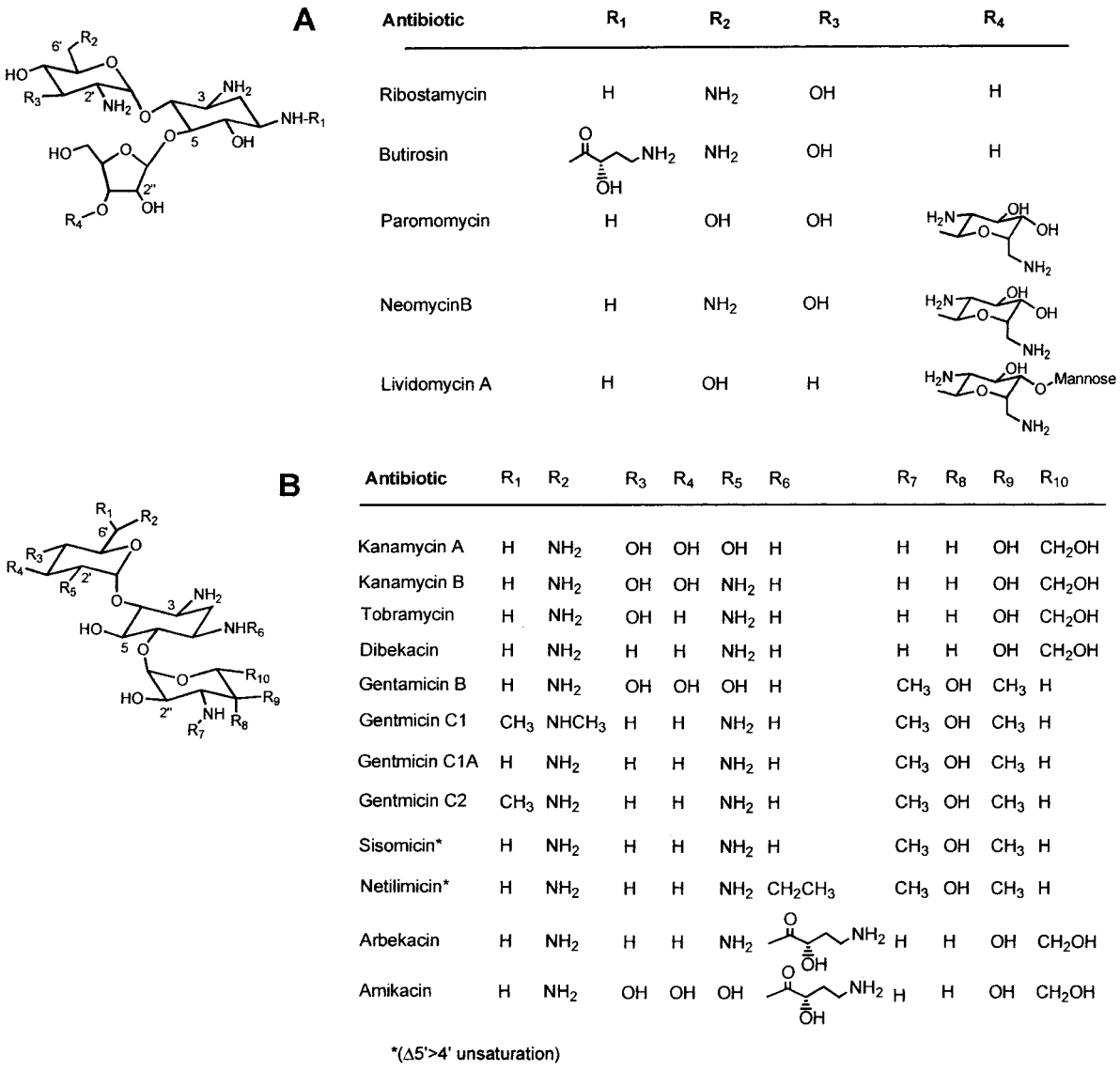

FIGURE 1: Structures of 4,5-disubstituted (A) and 4,6-disubstituted (B) aminoglycosides used in the study.

calculated for different ligands from fluorescence titrations are presented in Table 1. The slope of the plot of $\log(F_o\Delta F/F\Delta F_\infty)$ versus log [L] was unity for all ligands (Figure 2, inset), indicating the formation of a 1:1 complex between the homodimeric enzyme and ligand under these conditions. The maximum changes in intrinsic fluorescence of AAC(6′)-Iy and mutant forms varied greatly among acyl-coenzyme A's and aminoglycosides. These changes were dependent on the structure rather than the association constant of individual ligands. The K_a value for lividomycin A, in the presence of a saturating concentration of acetyl-CoA, was 8-fold higher than for lividomycin A alone (Table 1). However, no change in fluorescence was observed upon the addition of acetyl-CoA to the enzyme in the presence of a saturating concentration of lividomycin A. The thermodynamic parameters of binding of tobramycin and lividomycin A, calculated from a van't Hoff analysis, are listed in Table 2.

Isothermal Titration Calorimetry. (A) Binding of Aminoglycosides to Wild-Type and Mutant Forms of AAC(6′)-Iy Aminoglycoside 6′-N-Acetyltransferase. Binding interactions between a number of aminoglycosides and the wild-type enzyme were studied by isothermal titration calorimetry. The thermodynamic binding parameters are presented in Table 3, and a representative ITC profile of tobramycin binding to AAC(6′)-Iy is shown in Figure 3. Among the 4,6-disubstituted aminoglycosides (Figure 1B), tobramycin, kanamycin B, and dibekacin exhibited high K_a and $-\Delta H$ values. Although association constants of sisomicin and netilmicin were in the same range, their binding enthalpies were significantly lower. The lowest association constants were determined for gentamicin, kanamycin A, and amikacin, and they also exhibited correspondingly lower $-\Delta H$ values. The product of the AAC(6′)-Iy reaction, 6′-acetyltobramycin, binds to the enzyme poorly, with a calculated K_a value of 2.1×10^4 M^{-1}. The values of ΔH and $T\Delta S$ for 6′-acetyltobramycin binding could not be obtained with certainty because of the low affinity and large standard error associated with ΔH.

Ribostamycin was found to be the best ligand among 4,5-disubstituted aminoglycosides (Figure 1A). The association constant of this antibiotic was 112×10^4 M^{-1} with a $-\Delta H$ value of 19.9 kcal mol^{-1}. Butirosin interacted with almost similar affinity, but it exhibited a lower $-\Delta H$ value (12.6

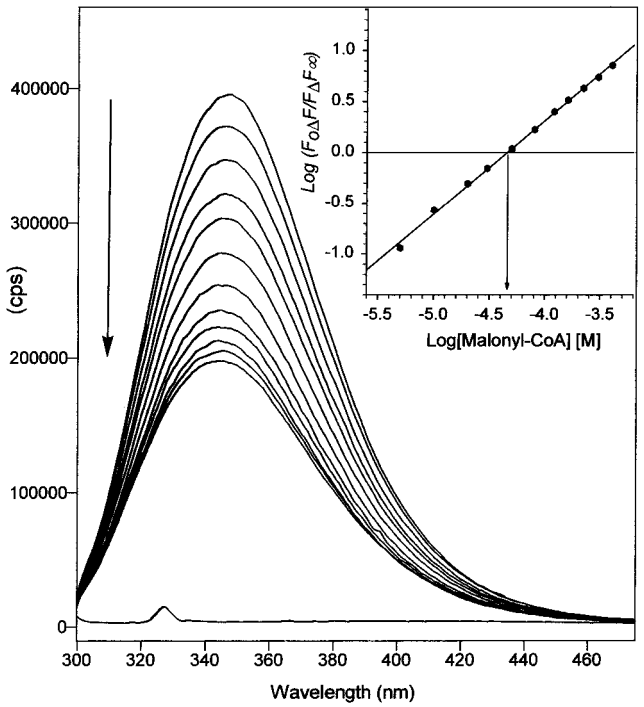

FIGURE 2: Titration of wild-type AAC(6′)-Iy with malonyl-CoA at 25 °C. The AAC(6′)-Iy (2.5 μM, 2 mL) with malonyl-CoA. The lines in the direction of the arrow are overlaid fluorescence spectra in the absence of malonyl-CoA and in the presence of 5, 10, 20, 30, 50, 50, 120, 160, 220, 300, and 400 μM malonyl-CoA and buffer and ligand baseline in the absence of protein. Inset: Graphical representation of the determination of the association constant.

Table 1: Association Constants for Aminoglycosides and Coenzyme A Derivative Binding to AAC(6′)-Iy and Mutant Forms from Fluorescence Titrations at 25 °C

acyl-CoA	K_a ($\times 10^4$ M^{-1})[a]	aminoglycoside	K_a ($\times 10^4$ M^{-1})[a]
wild type			
acetyl-CoA	13.1 ± 0.7	tobramycin	12.3 ± 0.3
n-hexanoyl-CoA	11.9 ± 0.4	lividomycin A	3.9 ± 0.1
isobutyryl-CoA	8.5 ± 0.5	(with Ac-CoA)[b]	33.1 ± 2.0
isovaleryl-CoA	7.5 ± 0.1	sisomicin	14.1 ± 0.8
malonyl-CoA	2.3 ± 0.1	ribostamycin	50.1 ± 4.8
Me-malonyl-CoA	3.1 ± 0.1	neomycin B	29.5 ± 1.5
		kanamycin B	7.1 ± 0.4
		kanamycin A	3.3 ± 0.1
		amikacin[c]	4.1 ± 0.1
C109A mutant			
acetyl-CoA	5.0 ± 0.1	ND[d]	
malonyl-CoA	1.5 ± 0.1		
C109A/C70A double mutant			
acetyl-CoA	7.9 ± 0.1	ND	
malonyl-CoA	1.9 ± 0.1		

[a] Uncertainties are the standard errors of the linear fit (Figure 2). [b] In the presence of a saturating amount of (bound) acetyl-CoA. [c] A decrease in intrinsic fluorescence intensity was observed upon titration. [d] ND, not detectable as intrinsic fluorescence is insensitive to aminoglycoside binding in these mutant enzymes.

kcal mol^{-1}) compared to ribostamycin. The K_a of neomycin B was lower than butirosin, but its enthalpy of binding was ~2 kcal mol^{-1} higher than that of the latter. Paromomycin and lividomycin A, both having a 6′-hydroxyl group, are potent inhibitors of AAC(6′)-Iy and are not O-acetylated by acetyl-CoA (*13*). Paromomycin showed the lowest association constant and $-\Delta H$ value among 4,5-disubstituted aminoglycosides, and the K_a and $-\Delta H$ values of lividomycin A were found to be higher than those of paromomycin. The enzyme, saturated with acetyl-CoA, was titrated with lividomycin A to determine the effect of the presence of enzyme-bound acetyl-CoA on the thermodynamics of aminoglycoside recognition. Figure 4 shows the binding isotherms of lividomycin A in the presence and absence of acetyl-CoA. The K_a of lividomycin A is significantly enhanced (to 154 $\times$ 10^4 M^{-1} from 21 $\times$ 10^4 M^{-1}) in the presence of bound acetyl-CoA.

Table 2: Thermodynamic Parameters of Binding of Aminoglycosides to AAC(6′)-Iy from van't Hoff Analysis

ligand	temp (°C)	K_a ($\times 10^4$ M^{-1})[a]	$-\Delta G$ (kcal mol^{-1})[a,b]	$-\Delta H$ (kcal mol^{-1})[a,b]	$-T\Delta S$ (kcal mol^{-1})[a,b]
tobramycin	10	41.69 ± 1.7			
	15	27.54 ± 0.8			
	20	15.32 ± 0.7			
	25	12.30 ± 0.3	6.92	15.5 ± 1.2	8.61
	30	8.32 ± 0.20			
	35	3.81 ± 0.15			
lividomycin A	10	9.88 ± 0.35			
	15	6.46 ± 0.09			
	20	5.69 ± 0.08			
	25	3.90 ± 0.07	6.24	10.8 ± 0.8	4.52
	30	3.02 ± 0.08			
	35	1.89 ± 0.03			

[a] Uncertainties are standard errors of the linear fit. [b] Values determined at 298 K.

Table 3: Thermodynamic Parameters Obtained from Calorimetric Titration of AAC(6′)-Iy with Different Aminoglycosides[a]

ligand	K_a ($\times 10^4$ M^{-1})	$-\Delta G$ (kcal mol^{-1})	$-\Delta H$ (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
wild-type AAC(6′)-Iy				
kanamycin B	13.4	7.0	13.0	6.0
tobramycin	22	7.3	12.1	4.8
dibekacin	28	7.4	12.8	5.4
gentamicin C	4.9	6.4	8.1	1.7
kanamycin A	6.3	6.6	9.6	3.3
amikacin	6.8	6.6	5.7	−0.9
sisomicin	18	7.2	8.9	1.7
netilmicin	20	7.3	6.0	−1.3
ribostamycin	112	8.3	19.9	11.6
butirosin	91	8.1	12.6	4.5
neomycin B	32	7.5	14.7	7.2
paromomycin	9.2	6.8	7.5	0.7
lividomycin A	21	7.3	9.4	2.1
lividomycin A[b]	154	8.5	10.6	2.1
Ac-tobramycin	~2.1	~5.9	ND[c]	ND
C109A mutant				
tobramycin	17	7.1	13.4	6.3
C109A/C70A mutant				
tobramycin	~1.4	~5.6	ND	ND

[a] Determined at 300 K. Error values: K_a, 1−6%; ΔH, 1−4%; ΔG, ~1%; $T\Delta S$, 2−7%; n, <2%. [b] In the presence of bound acetyl-CoA. [c] ND, not detectable.

The binding of tobramycin to the two mutant enzymes (C109A and C70A/C109A) was monitored by ITC to determine the importance of C109 and C70 in the binding of aminoglycosides. Compared to the wild-type enzyme, no significant change in the binding free energy (ΔG) was observed for the single mutant (C109A), and only a marginal increase in the $-\Delta H$ value was observed. In contrast, the mutation of the second Cys residue, using the C70A/C109A enzyme, significantly affected the affinity of the enzyme for tobramycin (Table 3).

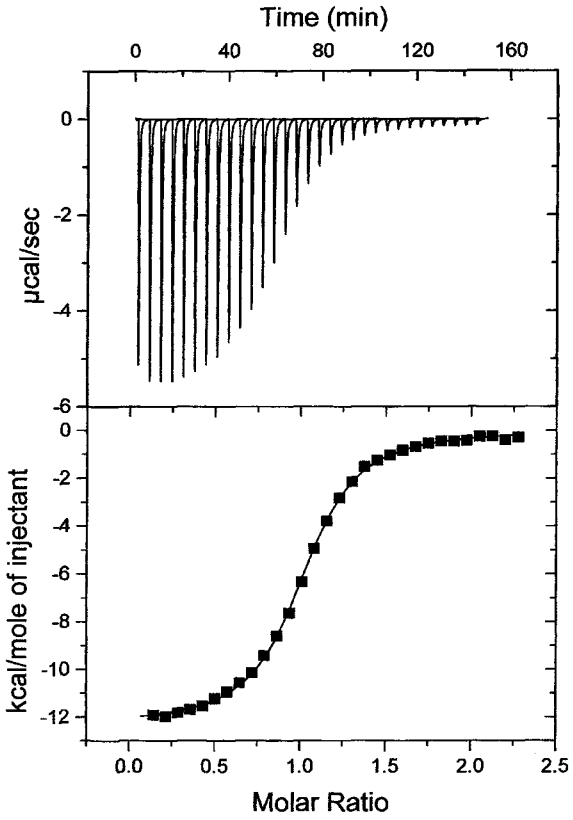

FIGURE 3: ITC profile of wild-type AAC(6′)-Iy (115 μM) with tobramycin (5 mM) at 27 °C. Top: data obtained for 30 automatic injections, 3 μL each of tobramycin. Bottom: the integrated curve showing experimental points (■) and the best fit (—). The experimental conditions are described in Experimental Procedures.

Table 4: Thermodynamic Parameters Derived from Calorimetric Titration of AAC(6′)-Iy and Its Two Mutants with Acyl-Coenzyme A Derivatives[a]

ligand	K_a ($\times 10^4$ M^{-1})	$-\Delta G$ (kcal mol^{-1})	$-\Delta H$ (kcal mol^{-1})	$-T\Delta S$ (kcal mol^{-1})
wild-type AAC(6′)-Iy				
acetyl-CoA	4.5	6.4	17.5	11.1
malonyl-CoA	3.5	6.2	8.1	1.9
isobutyryl-CoA	3.2	6.2	20.6	14.4
desulfo-CoA	4.1	6.3	11.1	4.8
C109A mutant				
acetyl-CoA	4.0	6.3	19.0	12.7
C109A/C70A mutant				
acetyl-CoA	3.9	6.3	18.7	12.4

[a] Determined at 300 K. Error values: K_a, 1−6%; ΔH, 1−4%; ΔG, ~1%; $T\Delta S$, 2−7%; n, <2%.

(B) Thermodynamic Binding Parameters of Acyl-CoA's. Table 4 presents the thermodynamic binding parameters obtained from the interactions of acyl-coenzymes with the wild-type and mutant enzymes. The $-\Delta H$ value for binding of acetyl-CoA to the wild-type enzyme was ~1.5 kcal mol^{-1} less than those determined with the mutant enzymes, but the entropy loss on binding was also less, resulting in the ΔG values remaining the same. The K_a of isobutyryl-CoA was slightly lower than that of acetyl-CoA, but its $-\Delta H$ was found to be 3.1 kcal mol^{-1} greater than the latter. The $-\Delta H$ of malonyl-CoA binding was significantly lower than the other analogues, yet its association constant was comparable to that of isobutyryl-CoA. Desulfo-CoA, one of the product analogues, binds to the enzyme with a K_a value identical to that of acetyl-CoA, but the $-\Delta H$ value for desulfo-CoA was 6.4 kcal mol^{-1} lower than for acetyl-CoA.

(C) Determination of ΔC_p. ITC binding studies with tobramycin and the wild-type enzyme were performed at four different temperatures (15, 22, 29, and 36 °C). The results are presented in Figure 5. The value of $-\Delta H$ increased from 7.1 kcal mol $^{-1}$ (at 15 °C) to 15.7 kcal mol^{-1} (at 36 °C). However, the ΔG slightly decreased with increasing temperature because of the larger increase in unfavorable entropy as the temperature increased. The ΔC_p determined from the binding of tobramycin to the wild-type enzyme was −410 cal mol^{-1} K^{-1}.

DISCUSSION

Enzymatic modification of aminoglycosides is the most prevalent mechanism of resistance to these antibiotics among microbial pathogens. In addition to the known AAC(1), AAC(2′), AAC(3), and AAC(6′) acetyltransferases, a new member of aminoglycoside *N*-acetyltransferases, termed AAC(4′′′), has been recently identified that confers resistance toward amikacin and arbekacin by acetylating the amine function of the N^1-aminohydroxybutyryl substituent of these aminoglycosides in methicillin-resistant *Staphylococcus aureus* (*16*). The 6′-amino group is one of the most important chemical determinants for the antibacterial activity of aminoglycosides, and not surprisingly, the aminoglycoside 6′-*N*-acetyltransferases are widespread among bacterial pathogens, with at least 18 distinct members of the major AAC(6′)-I subfamily being identified to date (*2*). Several kinetic and mechanistic studies have been reported on aminoglycoside *N*-acetyltransferases, and all reveal that they use a sequential kinetic mechanism (*13*, *17*−*22*). However, there have been no thermodynamic studies of the binding of either aminoglycosides or acyl-CoA derivatives to any aminoglycoside acetyltransferase to date. The present study provides a thermodynamic basis for aminoglycoside and acyl-CoA recognition by AAC(6′)-Iy.

The intrinsic tryptophan fluorescence of proteins can be exploited to obtain equilibrium ligand binding data. The *S. enterica* AAC (6′)-Iy has three tryptophan residues (W14, W22, and W103) that are completely conserved among the AAC (6′)-I subfamily, and W103 lies in the predicted acetyl-CoA binding domain (*13*). The quenching of fluorescence intensity upon the addition of acyl-CoA derivatives suggests that one or more tryptophan residues in AAC (6′)-Iy, and its mutants, are either directly involved in acyl-CoA binding or are in the vicinity of the binding pocket. The K_a values of acyl-CoA derivatives obtained from fluorescence titrations of the wild-type and mutant enzymes, within the experimental uncertainty, are in agreement with the values obtained from ITC measurements. Mutation of cysteine residues 109 and 70 to alanine did not have any significant effect either on acyl-CoA binding or on the intrinsic fluorescence properties of the enzymes. In the case of aminoglycosides, the increase in intrinsic fluorescence intensity upon aminoglycoside binding indicates a change in the microenvironment of one or more Trp residue(s) upon binding, either due to direct interactions with the aminoglycoside or indirectly due to changes in the environment of the tryptophan residues that result from aminoglycoside binding. The latter may be particularly important if sulfhydryl groups are present in the vicinity of tryptophan residues in the active site, since

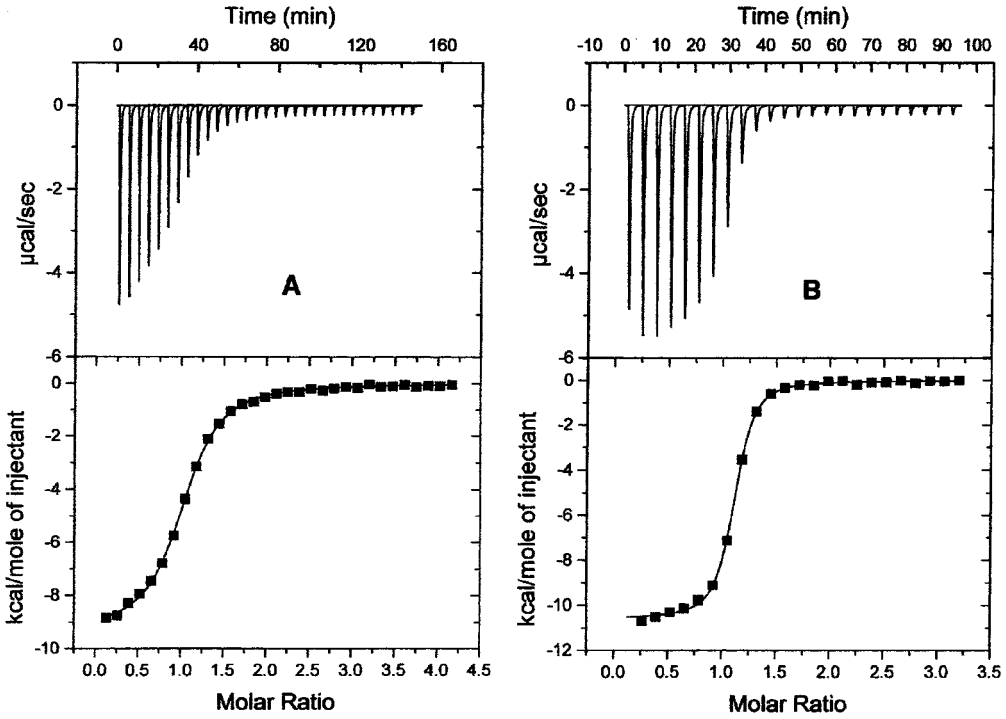

FIGURE 4: ITC profiles of (A) wild-type AAC(6′)-Iy (85 μM) with lividomycin A (5 mM) and (B) the wild-type AAC(6′)-Iy−acetyl-CoA binary complex with lividomycin A (5 mM), at 27° C. AAC(6′)-Iy (85 μM) was preincubated with 1 mM acetyl-CoA. (A) and (B) were performed under identical experimental conditions.

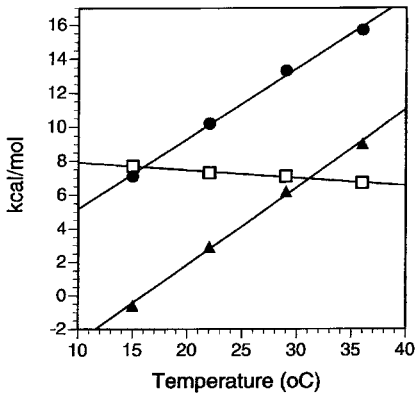

FIGURE 5: Temperature dependence of $-\Delta H$ (●), $-\Delta G$ (□), and $-T\Delta S$ (▲) for the binding of tobramycin (5 mM) to the wild-type AAC(6′)-Iy as determined by ITC.

sulfhydryl groups are known to have a profound influence on tryptophan fluorescence. This idea is supported by two independent observations; (i) previous kinetic and inactivation studies on wild-type and Cys → Ala mutant enzymes implicate the involvement of Cys residues in aminoglycoside binding and (ii) mutation of the completely conserved cysteine residue (C109A) results in the intrinsic protein fluorescence being insensitive to aminoglycoside binding. The latter is observed even though the C109A mutant enzyme exhibits steady-state kinetic parameters for substrates that, while altered, demonstrate the enzyme to be catalytically competent. Titration of the wild-type enzyme with amikacin, in contrast to other aminoglycosides, resulted in modest quenching of tryptophan fluorescence, while other N^1-substituted aminoglycosides (butirosin and netilmicin) did not have any significant effect on intrinsic fluorescence. This suggests that N^1-substituted aminoglycosides are binding in an orientation different from the corresponding N^1-unsubstituted aminoglycosides. Indeed, these differences in the binding of N^1-substituted and -unsubstituted aminoglycosides are reflected in the thermodynamic parameters determined in calorimetric studies (see below). The polar aminohydroxybutyryl moiety of amikacin may be extended toward one of the tryptophan residues, resulting in the observed quenching of fluorescence. The K_a values for aminoglycosides, obtained from fluorescence titrations, within the experimental uncertainties, are comparable to those obtained independently from ITC studies. The calculated van't Hoff enthalpies (ΔH_{vH}) obtained from the fluorescence studies using tobramycin and lividomycin A were similar to calorimetric enthalpy (ΔH_{cal}), with $\Delta H_{cal}/\Delta H_{vH}$ ratios between 0.8 and 0.9, compared to the expected value of 1.0.

The ITC data obtained with the enzyme and a panel of aminoglycosides highlight some of the structural requirements for binding. There is a good correlation between the steady-state kinetic parameters (*13*) and the thermodynamic parameters for the subset of mainly 4,6-disubstituted aminoglycosides, for which both values available. Overall, an increase in K_a and $-\Delta H$ values was observed with increasing *V/K* and *V* values, respectively. However, the K_m values alone did not exhibit any strict correlation with thermodynamic parameters though an inverse relationship was observed for a subset of aminoglycosides (amikacin, kanamycin B, sisomicin, and netilmicin). Moreover, the kinetic parameters, as observed earlier, were also dependent on the identity of the acyl-CoA derivatives (*13*). The binding of aminoglycosides to the enzyme is enthalpically driven with a broad range of affinities. Among 4,6-disubstituted aminoglycosides (Figure 1B), kanamycin B, dibekacin, and tobramycin exhibited the highest $-\Delta H$ values, and these values were comparable for all three sugars. The structures of these three substrates differ only in their 3′- and 4′-substituents. Apparently, the hydroxyl groups at these positions are not critical

for binding to the acetyltransferase, as removal of either the 3′- (as in tobramycin) or both 3′- and 4′-hydroxyls (as in dibekacin) does not significantly affect the binding enthalpies or affinities. Sisomicin and netilmicin both contain a 4′,5′ double bond and exhibit a similar affinity for the enzyme compared to kanamycin B, dibekacin, and tobramycin. Interestingly, the $-\Delta H$ values of sisomicin and netilmicin are 3−5 kcal/mol^{-1} less negative than the former three, but this is compensated for by a less unfavorable entropic contribution to binding. Kanamycin A differs from kanamycin B only at the 2′-position. Replacement of the 2′-amino group in kanamycin B by the 2′-hydroxyl group in kanamycin A results in a 3.4 kcal mol^{-1} decrease in the $-\Delta H$ value. This may reflect the ability of di- or triprotonic amino groups to engage in one or more hydrogen bonds than the monoprotonated hydroxyl group. Substitution at the N^1-position, with either an ethyl group as in netilmicin, or a bulky aminohydroxybutyryl group, as in amikacin, resulted in a decrease of 3 and 4 kcal mol^{-1} in the $-\Delta H$ value compared to their N^1-unsubstituted homologues sisomicin and kanamycin A, respectively. The less unfavorable entropy associated with the binding of these substrates (amikacin and netilmicin) results in their overall ΔG remaining unchanged. The favorable entropic contribution to the binding of netilmicin and amikacin suggests that the N^1 substituents of these substrates may be involved in hydrophobic interactions with residues in the enzyme active site.

Among the 4,5-disubstituted aminoglycosides tested (Figure 1A), ribostamycin was the best ligand (Table 3). The N^1-substituted ribostamycin derivative, butirosin, exhibited comparable affinity, but as observed with the 4,6-disubstituted aminoglycosides, the $-\Delta H$ value of butirosin decreased by 7.3 kcal mol^{-1} with a concomitant increase in favorable $T\Delta S$ value (7.1 kcal mol^{-1} more positive than ribostamycin). The addition of a hexose ring at the 3″-position, as in neomycin B, reduces the affinity and lowers the $-\Delta H$ of binding by 5.2 kcal mol^{-1}, suggesting that the active site of the enzyme poorly accommodates a hexose ring at this position. Paromomycin differs from neomycin B only by the replacement of the 6′-amino group of neomycin B with a 6′-hydroxyl group in paromomycin. This single difference reduces the K_a by 3−4-fold predominantly due to a decrease of the $-\Delta H$ value by 7.2 kcal mol^{-1}. As discussed above in comparing kanamycins A and B, we suggest that this is the result of the 6′-amino group being involved in multiple hydrogen-bonding interactions with the enzyme. Finally, the addition of an additional mannose residue to the 4‴-position, as in lividomycin A, contributes favorably to both K_a and ΔH.

The values of n for all the glycosides tested were found to be close to 1 using the native molecular mass of the enzyme, which is a homodimer. This stoichiometry indicates that either only one of two subunits of the dimer is capable of binding aminoglycoside or the active site is located at the interface of the two subunits. Steady-state kinetic evidence favors the former interpretation, since many of the aminoglycosides exhibit non-Michaelis−Menten kinetics, reminiscent of half-of-the-sites reactivity in an asymmetric homodimer (*13*).

The thermodynamics of acyl-coenzyme A binding to the enzymes are somewhat different from those obtained with the aminoglycosides. Acetyl-CoA and isobutyryl-CoA exhibited large negative binding enthalpies (−17.5 and −20.6 kcal mol^{-1}, respectively) but also large values of $-T\Delta S$. The presence of a charged carboxylate group in malonyl-CoA dramatically reduced the $-\Delta H$ value to 8.1 kcal mol^{-1}. Though their affinities and overall binding free energies were almost identical, these acyl-CoA substrates exhibit over a 1000-fold difference in catalytic efficiency (*13*). Desulfo-CoA, a dead-end inhibitor of the enzyme, exhibits an identical K_a and intermediary ΔH values to other acyl-CoA's. The ITC-derived n values suggest that acyl-CoA's also bind to a single active site in the homodimer.

The ITC-derived binding parameters of the inhibitor lividomycin A to the binary acetyl-CoA−enzyme complex reveal important aspects of synergistic substrate binding by the enzyme. Lividomycin A and acetyl-CoA bind to independent, but adjacent, sites on the enzyme, but the affinity of lividomycin A is 8 times higher for the acetyl-CoA−enzyme complex compared to the free enzyme. A similar increased affinity was observed in fluorescence titration studies (Table 1). The enthalpy of lividomycin A binding to the binary complex was −1.2 kcal mol^{-1} more favorable than binding to the free enzyme, while $T\Delta S$ values remained the same (Table 3). This may be due to an altered and more favorable conformation of the acetyl-CoA−enzyme complex or the presence of an additional interaction between the two bound ligands. The observed 12-fold affinity difference between the aminoglycoside substrate and the 6′-acetylated product and the stronger binding of aminoglycosides to the enzyme−acetyl-CoA complex support the kinetic model previously proposed for AAC(6′)-Iy (*13*).

The thermodynamic analysis of the interaction between tobramycin and acetyl-CoA and the two mutant enzymes (C109A and C70A/C109A) highlights the role of C70 and C109 in substrate binding. Binding of acetyl-CoA to the enzyme was not significantly affected by either of these mutations (Table 4). The single mutant C109A does not appear to significantly perturb the binding parameters of tobramycin although this mutant enzyme exhibited altered catalytic properties toward aminoglycosides (*13*). However, aminoglycoside binding was significantly impaired by the second mutation (C70A/C109A). This observation also confirms and extends previous steady-state kinetic studies on aminoglycoside binding to the double mutant (*13*).

ITC studies using tobramycin have allowed us to determine the temperature dependence of $-\Delta H$ and $-T\Delta S$. The temperature dependence of ΔG is very small (Figure 5). The ΔC_p was found to be −410 cal mol^{-1} K^{-1}, which is larger than the values reported for lectin−carbohydrate interactions (∼100 cal mol^{-1} K^{-1}) but smaller than those reported for antibody−carbohydrate (−1200 cal mol^{-1} K^{-1}) (*23*) and protein−protein interactions (−1500 cal mol^{-1} K^{-1}) (*24*). The ΔC_p obtained for the binding of UDP-*N*-acetylglucosamine to UDP-*N*-acetylglucosamine enolpyruvyltransferase is comparable to that of tobramycin binding to AAC(6′)-Iy (*25*). Changes in the solvent-accessible surface area upon binding have been proposed to be the primary contributor to negative ΔC_p values (*26*, *27*). Conformational changes and hydrophobic interactions due to the presence of aromatic amino acids are thought to contribute to the relatively higher ΔC_p value of antibody−carbohydrate binding. Samlad et al. (*25*) demonstrated that the experimentally determined ΔC_p of UDP-*N*-acetylglucosamine binding was greater than the

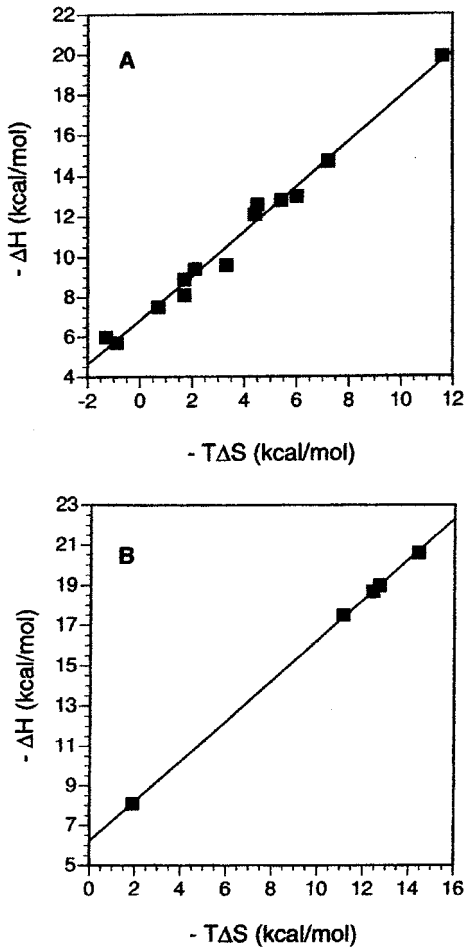

FIGURE 6: Plots of ITC-derived $-\Delta H$ versus $-T\Delta S$ for the binding of (A) aminoglycosides to the wild-type AAC(6′)-Iy and (B) acyl-CoA derivatives to the wild-type and mutant-type AAC(6′)-Iy.

value calculated from the changes in solvent-accessible surface area, suggesting that other factors significantly influenced the heat capacity of protein−ligand interactions.

Enthalpy−entropy compensation plots have previously been described for carbohydrate interactions with lectins (*28*) and antibodies (*29*) and attributed to solvent reorganization upon ligand binding and to the changes in rotational degrees of freedom of the ligand (*30*, *31*). In the present study, we suggest that the more numerous and favorable the enthalpic interactions between substrates and the acetyltransferase, the more rigidly aligned and constrained are both the substrates and the side chains of the amino acids that interact with the substrates in the active site. This leads to more conformational restrictions, both locally among the participating active site residues and globally, that would result in the more unfavorable entropic terms associated with binding. These conformational restrictions result in the observed compensations. Plots of the $-\Delta H$ versus $-T\Delta S$ values at 300 K for aminoglycosides and coenzyme A derivatives obtained with AAC(6′)-Iy exhibit a similar compensatory nature (Figure 6). These plots show an essentially linear relationship with a slope of 1.14 for the aminoglycosides (correlation coefficient 0.98) and 1.0 for the coenzymes (correlation coefficient 0.99). The slopes of enthalpy−entropy compensation plots reported for lectin−carbohydrate interactions are greater than unity, in contrast to antibody−carbohydrate interactions where the slope is often less than unity. A slope greater than unity indicates that the free energy of binding is predominantly driven by enthalpy, while a slope less than unity indicates dominant entropy contributions.

There are no three-dimensional structures of aminoglycosides bound to any aminoglycoside acetyltransferase, making a detailed interpretation of substrate binding difficult and speculative. However, the three-dimensional structures of two aminoglycoside acetyltransferase−CoA complexes, including both the 3-*N*-acetyltransferase from *Serratia marascens* (*32*) and the 6′-*N*-acetyltransferase from *Enterococcus faecalis* (*33*), have been reported and reveal that the overall protein fold is very similar despite almost no sequence similarity. Similarly, the position of CoA bound to these proteins is essentially superimposable. This suggests that the structure of the AAC(6′)-Iy could be modeled using these structures as a scaffold in order to allow residues that possibly contribute to aminoglycoside binding to be identified. Given the extremely small size of these enzymes (145−215 amino acids), the ability to tightly bind and regioselectively acetylate one of the four or five amino groups on a substrate like tobramycin is remarkable. This is especially true since the bona fide physiological role of many, if not all, of these enzymes, including the chromosomally encoded *S. enterica* AAC(6′)-Iy studied here, is unknown.

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