Kinetic and Mutagenic Characterization of the Chromosomally Encoded *Salmonella enterica* AAC(6′)-Iy Aminoglycoside *N*-Acetyltransferase†

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ABSTRACT: The chromosomally encoded aminoglycoside *N*-acetyltransferase, AAC(6′)-Iy, from *Salmonella enterica* confers resistance toward a number of aminoglycoside antibiotics. The structural gene was cloned and expressed and the purified enzyme existed in solution as a dimer of ca. 17 000 Da monomers. Acetyl-CoA was the preferred acyl donor, and most therapeutically important aminoglycosides were substrates for acetylation. Exceptions are those aminoglycosides that possess a 6′-hydroxyl substituent (e.g., lividomycin). Thus, the enzyme exhibited regioselective and exclusive acetyltransferase activity to 6′-amine-containing aminoglycosides. The enzyme exhibited Michaelis−Menten kinetics for some aminoglycoside substrates but "substrate activation" with others. Kinetic studies supported a random kinetic mechanism for the enzyme. The enzyme was inactivated by iodoacetamide in a biphasic manner, with half of the activity being lost rapidly and the other half more slowly. Tobramycin, but not acetyl-CoA, protected against inactivation. Each of the three cysteine residues (C70, C109, C145) in the wild-type enzyme were carboxamidomethylated by iodoacetamide. Cysteine 109 in AAC(6′)-Iy is conserved in 12 AAC(6′) enzyme sequences of the major class I subfamily. Surprisingly, mutation of this residue to alanine neither abolished activity nor altered the biphasic inactivation by iodoacetamide. The maximum velocity and V/K values for a number of aminoglycosides were elevated in this single mutant, and the kinetic behavior of substrates exhibiting linear vs nonlinear kinetics was reversed. Cysteine 70 in AAC(6′)-Iy is either a cysteine or a threonine residue in all 12 AAC(6′) enzymes of the major class I subfamily. The double mutant, C109A/C70A, was not inactivated by iodoacetamide. The double mutant exhibited large increases in the K_m values for both acetyl-CoA and aminoglycoside substrates, and all aminoglycoside substrates exhibited Michaelis−Menten kinetics. Solvent kinetic isotope effects on V/K were normal for the WT enzyme and inverse for the double mutant. We discuss a chemical mechanism and the likely rate-limiting steps for both the wild-type and mutant forms of the enzyme.

Aminoglycosides are natural, or semisynthetic, molecules consisting of a central six-membered aminocyclitol ring linked to two or more amino sugars by glycosidic bonds. These broad-spectrum antibacterial compounds were among the first antibiotics discovered and are used in the treatment of severe Gram-positive and Gram-negative infections, often in combination with β-lactams or fluoroquinolones. Their mechanism of action is the inhibition of bacterial protein synthesis by binding to the small ribosomal subunit. Aminoglycosides additionally display pleiotrophic effects on the bacterial cell and have a concentration-dependent bactericidal activity attributed to irreversible binding to the ribosome and damage to the membrane (*1*, *2*).

Resistance to aminoglycosides can result from three causes: (i) decreased intracellular accumulation of the drug by changes in the permeability of the outer membrane (*3*), alteration of specific cytoplasmic membrane transport systems (*4*, *5*), or active efflux (*6*, *7*), (ii) modification of the target by 16S RNA mutation or ribosomal protein modification (*3*), and (iii) enzymatic modification of the drug (*8*, *9*), which is the most common resistance mechanism. Three activities can cause covalent modification of the aminoglycosides: *O*-phosphorylation, *O*-nucleotidylation, and *N*-acetylation. Expression of genes encoding these various enzymes can result from acquisition of foreign DNA (*10*) or from overexpression of a housekeeping gene with modifying activity (*11*−*13*). Aminoglycoside *N*-acetyltransferases (AAC) use acetyl-coenzyme A (acetyl-CoA) as one substrate and transfer the acetyl group to an aminoglycoside amine function. There are four classes of acetyltransferase: AAC(1), AAC(2′), AAC(3), and AAC(6′), designated according to the site of the regioselective modification on the aminogly-

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coside. Members of the AAC(6′) class are the most common in nature and of particular importance since they modify aminoglycosides used most often in antibacterial chemotherapy, including amikacin, gentamicin, netilmicin, sisomicin, and tobramycin. The classes of AAC are further subdivided according to their substrate specificity. Four types of AAC(6′) have been distinguished; for example, type I confers resistance to amikacin but not to gentamicin, whereas type II inactivates gentamicin but not amikacin (*14*).

There have been few, even rudimentary, mechanistic studies on aminoglycoside-modifying enzymes. The most complete, preceding the explosion in the number and types of recognized aminoglycoside modifying enzymes, are due to Northrop and co-workers in the early 1980s, who focused on gentamicin acetyltransferase AAC(3)-I (*15*, *16*) and kanamycin acetyltransferase AAC(6′)-IV (*17*, *18*). Their kinetic results on AAC(3)-I are consistent with a random bi-bi mechanism. More recently, the Wright group has reported an analysis of the chromosomally encoded AAC(6′)-Ii from *Enterococcus faecium* and shown that this enzyme exhibits broad substrate specificity for aminoglycosides (*19*).

Isolation, from a stool culture of a single patient, of two *Salmonella enterica* serotype Enteritidis strains BM4361, susceptible to aminoglycosides, and BM4362, resistant to tobramycin and dibekacin, led to the identification of the chromosomal *aac(6′)-Iy* gene (*20*). This gene was cryptic in the susceptible parental strain but was expressed in the derived BM4362 strain as a consequence of a 60-kb deletion leading to a transcriptional fusion. Aminoglycoside acetyltransferase activity in crude extracts of BM4362 was consistent with the production of a 6′-*N*-aminoglycoside acetyltransferase of type I [AAC(6′)-I]. The deduced AAC(6′)-Iy sequence consists of 145 amino acids and belongs to the major AAC(6′)-I subfamily defined on the basis of amino acid sequence homology (*21*). This family includes, among others, AAC(6′)-If, a plasmid-encoded enzyme in *Enterobacter cloacae*, AAC(6′)-Ic in *Serratia marcescens*, and AAC(6′)-Ig in *Acinetobacter haemolyticus*.

In this paper, we report the cloning and heterologous overexpression of the *S. enterica aac(6′)-Iy* gene and the purification of the encoded 6′-*N*-acetyltransferase. We have studied the substrate specificity of AAC(6′)-Iy for various aminoglycosides and CoA thioester analogues and determined the kinetic mechanism of this enzyme. The determination of solvent kinetic isotope effects, site-directed mutagenesis, and inactivation of the enzymatic activity by iodoacetamide led us to propose a chemical mechanism for AAC(6′)-Iy.

MATERIALS AND METHODS

Cloning and Overexpression of the aac(6′)-Iy Gene. Two oligonucleotides, O1, 5′-GAATTCCATATGGACATCAGGC-3′, complementary to the amino-terminal coding strand of *aac(6′)-Iy*, and O2, 5′-CGGAATTCA**ATGGTGATGGTGATGGTG**ACAACGCTTTCGG-3′, complementary to the carboxy-terminal noncoding strands with the addition of a 6-histidine tag (bold characters) containing *Nde*I and *Eco*RI restriction sites (underlined), respectively, were synthesized (Unité de Chimie Organique, Institut Pasteur, Paris, France). Using this pair of primers and *Pfu* DNA polymerase (Stratagene, La Jolla, CA), the *aac(6′)-Iy* gene was amplified from pAT711 plasmid DNA (*26*) under standard PCR conditions in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer Cetus, Norwalk, CT). The amplification product was ligated into the pCR-blunt vector (Zero Blunt Kit, Invitrogen, San Diego, CA). The recombinant plasmid was purified using the Wizard miniprep DNA kit (Promega, Madison, WI), and the sequence of the insert was determined by the dideoxynucleotide chain termination method (*22*) using T7 DNA polymerase (Pharmacia Biotech, Saint-Quentin-en-Yvelines, France) and [α-^{35}S]dATP (400 Ci/mmol; Amersham Radiochemical Center). The *Nde*I−*Eco*RI digested insert was separated using the Sephaglas BandPrep Kit (Pharmacia Biotech) and ligated into a pET23a(+) expression vector (Novagen, Madison, WI) previously digested with the same restriction enzymes. The resulting recombinant plasmid, pAT791, was transformed into *Escherichia coli* BL21(DE3)pLysS competent cells (Novagen) and plated on brain heart infusion agar (BHIA) containing 50 μg/mL ampicillin. A single transformant was inoculated in 1 L of 2× Luria Broth (LB) containing 2% glycerol and ampicillin (100 μg/mL). In the late exponential phase the culture was induced using 0.6 mM isopropyl thiogalactopyranoside (IPTG) and grown for an additional 3 h at 37 °C. Analysis of the overexpressed *aac(6′)-Iy* gene product was carried out by SDS−PAGE using 8−25% Phastgels (PhastSystem, Pharmacia).

Mutagenesis. Two pairs of complementary primers, O3 (5′-CGAATAAAGGG**GC**TCGGG AAATGG-3′)/O4 (5′-CCATTTCCCGA**GC**CCCTTTATTCG-3′) and O5 (5′-CGATTATGTCAATGG**CG**CTGACAGTTCGCCC-3′)/O6 (5′-GGGCGAACTGTCA**GC**GCCATTGACATAATCG-3′), containing two mutations (bold characters) relative to the pAT791 sequence, were synthesized to mutagenize *aac(6′)-Iy* genes. Using the QuikChange Site Directed Mutagenesis Kit (Stratagene), pAT791 DNA as a template, and O3/O4 as primers, pAT792 containing the mutagenized *aac(6′)-Iy* gene encoding the AAC(6′)-Iy C109A mutant was produced. In a second step, pAT793 encoding a double mutant enzyme, AAC(6′)-Iy C109A/C70A, was obtained using pAT792 as a template and the O5/O6 primer pair. Nucleotide sequencing of the inserts in pAT792 and pAT793, transformation of *E. coli* BL21(DE3)pLysS, and all subsequent steps were performed as for the wild type (WT).

Protein Purification. Cells were recovered by centrifugation from 1 L of 2× LB[1] culture of *E. coli* BL21(DE3)-pLysS/pAT791-793, obtained as described above, and were resuspended in 100 mL of 50 mM Tris, pH 7.5, and 200 mM NaCl containing lysozyme (0.3 mg/mL) and protease inhibitors (two Complete cocktail tablets, Boehringer Mannheim). All subsequent steps were performed at 4 °C. After being stirred for 30 min, cells were lysed by ultrasonic disruption, and cell debris was removed by centrifugation at 19000 rpm for 30 min. The clear extract obtained after a 3 h dialysis of the supernatant against 50 mM Tris, pH 7.5, and 200 mM NaCl was applied to a column containing 100 mL Ni-NTA His.Bind resin (Novagen) equilibrated with the same solution. After the column was washed with equilibration buffer first lacking and then containing 30 mM imid-

[1] Abbreviations: IPTG, isopropyl thiogalactopyranoside; DTT, dithiothreitol; IAM, iodoacetamide; LB, Luria broth; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

azole, the protein was eluted with 50 mM Tris, pH 7.5, and 200 mM NaCl containing 300 mM imidazole. The fractions with the highest enzymatic activity were pooled, dialyzed against 50 mM Tris, pH 7.5, and 50 mM NaCl, and loaded onto a 100 mL fast-flow Q-Sepharose anion-exchange column (Pharmacia) which had been equilibrated with the same solution. The protein was eluted with a 0.05−1 M NaCl step gradient. The active fractions were pooled, concentrated (YM-10 membrane, Amicon), dialyzed against 50 mM Tris, pH 7.5, containing 100 μM EDTA and 100 μM DTT, and then analyzed by SDS−PAGE. The native molecular weight of the wild-type enzyme was determined by chromatography on a Superdex 75 gel filtration column (Pharmacia) and using molecular weight standards (Bio-Rad). Active, purified enzyme was stored at −20 °C with 20% glycerol.

Measurement of Enzyme Activity. Reaction rates were determined spectrophotometrically by measuring the increase in absorbance at 412 nm due to the formation of 5-thio-2-nitrobenzoate resulting from the reaction between the free sulfhydryl group of CoASH, generated by the aminoglycoside acetylating activity, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The reaction was monitored continuously on a UVIKON 931 spectrophotometer. Assay mixtures contained 50 mM HEPES, pH 7.5, and 0.8 mM DTNB in addition to substrates and inhibitors. Reactions were initiated by the addition of 10 μL of enzyme and carried out at 37 °C in a total volume of 0.6 mL. Initial velocity kinetic data were fitted using the programs of Cleland (*23*). Equation 1 was used to fit simple substrate saturation kinetics, where the concentration of one substrate was varied at a fixed and saturating (>10 × K_m) concentration of the other substrate. Equation 2 was used to fit initial velocity patterns, where the concentrations of both substrates were varied. Equations 3, 4, and 5 were used to fit competitive, uncompetitive, and noncompetitive inhibition, respectively.

$$v = V/(K_a + A) \quad (1)$$

$$v = VAB/(K_aB + K_bA + K_{ia}K_b + AB) \quad (2)$$

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (3)$$

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (4)$$

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (5)$$

where v represents measured reaction velocity, V is the maximal velocity, A and B are the concentrations of the substrates (antibiotic and coenzyme), K_a and K_b are the corresponding Michaelis−Menten constants, K_{ia} is the inhibition constant for substrate A, I is the concentration of the inhibitor, and K_{is} and K_{ii} are the slope and intercept inhibition constants for inhibitors, respectively.

Equation 6 was used to fit kinetic plots in which "substrate activation" was apparent from Lineweaver−Burk plots, where A is the concentration of the variable substrate, in all cases the aminoglycoside. The constants B, C, and D are collections of rate constants that depend on the kinetic mechanism and the resulting rate equation (see Supporting Information for derivation).

$$v = V(A^2 + DA)/(A^2 + BA + C) \quad (6)$$

Solvent Kinetic Isotope Effects. The solvent kinetic isotope effects on V and V/K were determined by measuring the initial velocity of the reaction with saturating concentrations of acetyl-CoA and varying concentrations of aminoglycoside, in H_2O or ca. 80% D_2O as solvent. Solvent deuterium isotope effects were calculated from the equation:

$$v = VA/[K_A(1 + II_{VK}) + A(1 + II_V)] \quad (7)$$

where I represents the fraction of isotope and I_{VK} and I_V are the isotope effects on V/K and $V - 1$, respectively.

Inactivation by Iodoacetamide and Protection by Substrates. Purified *N*-acetyltransferases were incubated at 25 °C in the absence or presence of 5 mM iodoacetamide (IAM) and additionally with either 1 mM tobramycin or 1 mM acetyl-CoA or 1 mM dithio-CoA. Aliquots (5 μL) were removed at various time points and added to a standard reaction mixture containing saturating concentrations of tobramycin and acetyl-CoA as substrates to assess the residual enzyme activity. The inactivation rate was expressed relative to the activity of the preincubated control enzyme.

Mass Spectrometry. Samples of purified enzymes (WT and C109A) incubated in the absence or presence of 5 mM IAM for 6 h were desalted by extensive dialysis against 50 mM ammonium bicarbonate. The molecular masses of the molecules were determined by electrospray ionization mass spectrometry (Unité de Chimie Structurale des Macromolécules, Institut Pasteur, Paris, France).

Materials. BHIA and LB were from Difco Laboratoires (Detroit, MI). *Nde*I and *Eco*RI restriction enzymes were purchased from New England BioLabs (Beverly, MA). Deuterium oxide, IAM, acetyl-CoA, CoA derivatives, DTNB, amikacin, kanamycin A, kanamycin B, butirosin, dibekacin, netilmicin, and ribostamycin were obtained from Sigma (St. Louis, MO). Lividomycin and sisomicin were from Laboratoire Roger Bellon (Neuilly sur Seine, France), apramycin and tobramycin were from Eli Lilly (Indianapolis, IN), and neomycin A and neomycin C were from Roussel UCLAF (Romainville, France).

RESULTS

Purification and Properties of Wild-Type Aminoglycoside-6′-N-acetyltransferase-Iy and Mutant Derivatives. A single fragment of the expected size for the *aac(6′)-Iy* gene was obtained by PCR using oligonucleotides O1/O2 and pAT711 DNA as a template. The nucleotide sequence of the PCR product was determined to be identical to the published *aac(6′)-Iy* (*26*). Overexpression of the gene in *E. coli* BL21(DE3)pLysS cells led to the production of approximately 20 mg of a soluble protein/L of culture, with an apparent molecular mass by SDS−PAGE in agreement with the predicted value of 17 173 Da. The two-step purification procedure yielded greater than 90% pure protein that eluted at an apparent molecular mass of 35 kDa on a Superdex 75 gel filtration column, suggesting that the enzyme existed as a dimer in solution. The two AAC(6′)-Iy mutant enzymes, AAC(6′)-Iy C109A and AAC(6′)-Iy C109A/C70A, were produced in the same manner after verification of the sequence of the inserts pAT792 and pAT793. For the three enzymes, an enzyme concentration-dependent tobramycin acetyltransferase activity was detected by spectrophotometric assay. Incubation of the enzymes with DTNB resulted in no

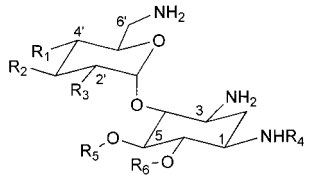
Table 1: Kinetic Parameters for Aminoglycoside and Acyl-CoA Analogues

	K_m (μM)	V_{max}[c]	V/K	K_m^2[b] (μM)	V_{max}^2[b,c]	V/K^2[b]
			AAC(6′)-Iy			
acyl-CoA						
acetyl-CoA	10 ± 2	930 ± 40	93			
propionyl-CoA[a]	270 ± 10	2070 ± 80	8			
malonyl-CoA	820 ± 130	890 ± 80	1			
butyryl-CoA	9300 ± >10000	1370 ± 1640	0.1			
aminoglycoside						
netilmicin[d]	8 ± 1	120 ± 20	15.0	300 ± 240	190 ± 10	0.6
tobramycin	66 ± 6	950 ± 20	14.4			
sisomicin[d]	12 ± 4	140 ± 20	11.7	290 ± 70	380 ± 10	1.3
neomycin C[d]	14 ± 7	160 ± 30	11.4	480 ± 80	770 ± 20	1.6
ribostamycin[d]	50 ± 15	400 ± 60	8.0	2200 ± 900	1480 ± 210	0.7
dibekacin[d]	30 ± 15	180 ± 50	6.0	580 ± 360	410 ± 30	0.7
kanamycin B	79 ± 6	265 ± 4	3.4			
amikacin[d]	50 ± 20	80 ± 25	1.6	610 ± 150	271 ± 7	0.4
kanamycin A	105 ± 6	126 ± 2	1.2			
neomycin A	530 ± 40	123 ± 3	0.2			
			AAC(6′)-Iy C109A			
acetyl-CoA	17 ± 1	1890 ± 20	111			
netilimicin	6 ± 0.6	700 ± 5	117			
tobramycin[d]	26 ± 3	1719 ± 70	66	140 ± 30	2460 ± 80	17
kanamycin B[d]	17 ± 2	2280 ± 40	134	58 ± 2	2570 ± 10	44
amikacin	75 ± 5	730 ± 15	10			
			AAC(6′)-Iy C109A/C70A			
acetyl-CoA	70 ± 10	1340 ± 100	19.2			
netilimicin	460 ± 40	440 ± 20	1.0			
tobramycin	540 ± 80	1320 ± 120	2.4			
kanamycin B	900 ± 40	1320 ± 30	1.5			
neomycin C	250 ± 10	510 ± 10	2.0			

[a] Aminoglycoside-independent hydrolysis of propionyl-CoA. [b] Values at high substrate concentrations. V_{max}^2 is the sum of the velocities at low and high concentrations. [c] Velocity units are ΔA_{412}/(min·mg) ($\times 10^3$). [d] Substrate exhibiting substrate activation.

loss of enzymatic activity. A slow, time-dependent loss of activity could be prevented by the inclusion of 100 μM DTT in the stock enzyme solutions.

Substrate Specificity of AAC(6′)-Iy. Initial velocities were determined spectrophotometrically at pH 7.5 and at 10 different concentrations of each substrate. The data were plotted as a double reciprocal plot and fitted using the programs of Cleland (*23*). Kinetic constants for acetyl-CoA and various CoA derivatives, determined at a saturating concentration of tobramycin, are presented in Table 1. Propionyl-CoA exhibited a rapid, tobramycin-independent rate of hydrolysis that was linearly proportional to enzyme, while the other acyl-CoA's exhibited very low rates of aminoglycoside-independent hydrolysis. Acetyl-CoA is the best of these substrates on the basis of relative V/K values and was used as acyl donor in subsequent experiments. The steady-state kinetic parameters for various aminoglycosides at saturating acetyl-CoA concentrations are summarized in Table 1. The structures of aminoglycosides listed in this table are shown in Figure 1. Aminoglycoside substrates can be separated into two groups on the basis of their kinetic behavior. Tobramycin, kanamycin A, kanamycin B, and neomycin A yielded linear double reciprocal plots, whereas netilmicin, sisomicin, neomycin C, ribostamycin, dibekacin, and amikacin exhibited linear kinetics at low substrate concentrations but rapidly increasing reaction velocities at higher aminoglycoside concentrations (Figure 2), suggesting substrate activation. For these substrates, two pairs of fitted kinetic constants are included in Table 1. On average, V^2 was 2.5 times higher than V^1, K^2 was 25 times higher than K^1, and V/K^2 was 10 times lower than V/K^1. No acetyltransferase activity could be detected using lividomycin or paromomycin, which have a hydroxyl group at the 6′ position. The steady-state kinetic behavior of AAC(6′)-Iy C109A was determined using tobramycin, netilmicin, amikacin, and kanamycin B, and the resulting kinetic constants are indicated in Table 1. The presence or absence of substrate activation for these aminoglycoside substrates was inverted relative to the results observed with the WT AAC(6′)-Iy:



Antibiotic	R_1	R_2	R_3	R_4	R_5	R_6
Neomycin A	OH	OH	NH_2	H	H	H
Kanamycin B	OH	OH	NH_2	H	H	3-deoxy-3-aminoglucose
Tobramycin	OH	H	NH_2	H	H	3-deoxy-3-aminoglucose
Kanamycin A	OH	OH	OH	H	H	3-deoxy-3-aminoglucose
Amikacin	OH	OH	OH		H	3-deoxy-3-aminoglucose
Dibekacin	H	H	NH_2	H	H	3-deoxy-3-aminoglucose
Sisomicin*	H	H	NH_2	H	H	sisamine
Netilimicin*	H	H	NH_2	CH_2CH_3	H	sisamine
Ribostamycin	OH	OH	NH_2	H	ribose	H
Neomycin C	OH	OH	NH_2	H	neobiosineC	H

* ($\Delta 5'>4'$ unsaturation)

FIGURE 1: Structures of aminoglycosides used in the study.

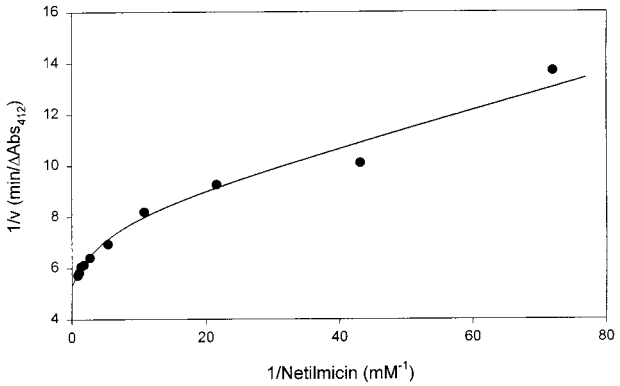


FIGURE 2: Double reciprocal plot of initial velocities versus [netilimicin] obtained at a fixed, saturating [acetyl-CoA] = 166 μM. The symbols are the experimentally determined values, while the smooth curve is a fit of the data to eq 2.

Table 2: Dead-End Inhibition Studies

inhibitor	variable substrate	K_{ii}	K_{is}	pattern
dithio-CoA	acetyl-CoA		15 ± 1	C
dithio-CoA	tobramycin	103 ± 10	28 ± 8	NC
lividomycin	acetyl-CoA	14 ± 2	17 ± 10	NC
lividomycin	tobramycin	4 ± 2	1.4 ± 0.6	NC

substrate activation was observed for tobramycin and kanamycin B, whereas netilmicin and amikacin exhibited linear reciprocal plots. The double reciprocal plots obtained for tobramycin, kanamycin B, netilmicin, and neomycin C with AAC(6′)-Iy C109A/C70A were all linear, and the resulting K_m values were very high. $K_{acetyl-CoA}$ was increased by 2- and 10-fold for the single and the double mutants, respectively (Table 1).

Kinetic Mechanism. The initial velocity pattern was determined using tobramycin and acetyl-CoA, at five different concentrations of each substrate. The double reciprocal plot observed was intersecting (figure in Supplementary Information), indicating a sequential kinetic mechanism. To determine if the kinetic mechanism was either ordered or random, alternative aminoglycoside kinetics and dead-end inhibition experiments were performed. Alternative antibiotic kinetics were determined using acetyl-CoA as the variable substrate at fixed, saturating concentrations of four different aminoglycosides. The double reciprocal plot of the data is presented as Supporting Information. The values of V/K for the acetyl-CoA varied modestly (over a 5.5-fold range) as a function of the identity of the aminoglycoside, with this change being exclusively as a result of changes in V rather than the K_m value for acetyl-CoA. Dead-end inhibition experiments were carried out using desulfocoenzyme A or lividomycin versus acetyl-CoA and tobramycin. The resulting inhibition patterns and corresponding inhibition constants are indicated in Table 2.

Solvent Kinetic Isotope Effects. Solvent kinetic isotope effects on acetyl transfer were determined by measuring initial velocities at pH 7.5, in both H_2O and approximately 80% D_2O. Reactions were performed at a fixed, saturating concentration of acetyl-CoA and at varying aminoglycoside concentrations. Two different aminoglycosides were examined, tobramycin and neomycin A, for the wild-type enzyme. The solvent kinetic isotope effects on V were identical, and equal to 1.27 ± 0.04, for both aminoglycosides. The solvent

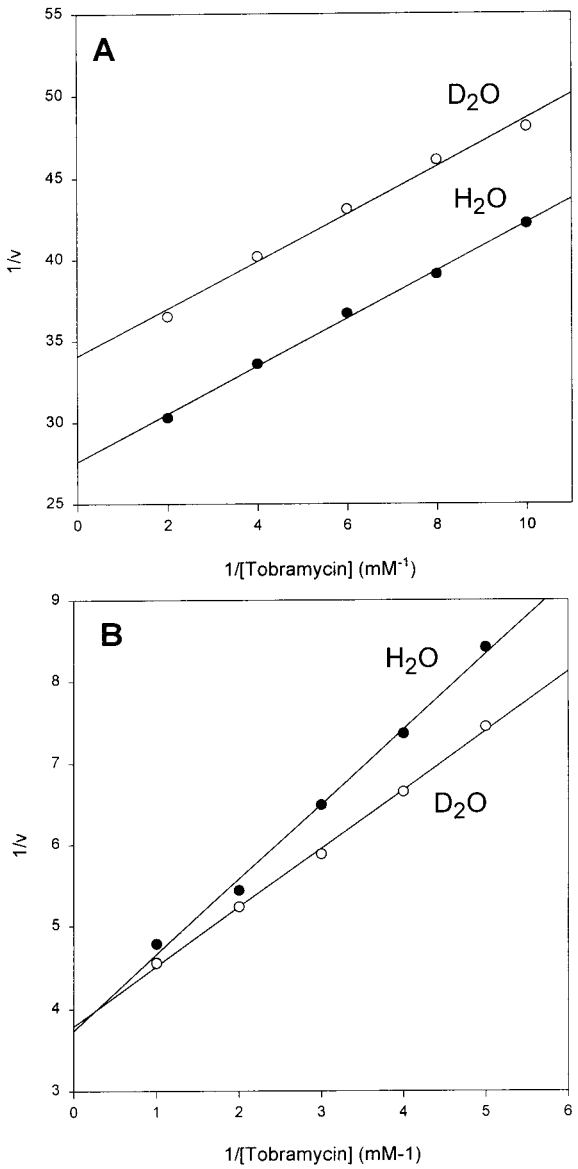


FIGURE 3: Solvent kinetic isotope effect for AAC(6′)-Iy and the C109A/C70A double mutant. The symbols are the experimentally determined values in H_2O (○) or ca. 80% D_2O (●), while the lines are fits of the data to eq 4. Tobramycin was the variable substrate, and acetyl-CoA was present at a saturating concentration of 166 μM using either the WT (A) or C109A/C70A double mutant (B).

kinetic isotope effects on V/K were different; no solvent kinetic isotope effect was observed on $V/K_{tobramycin}$ (Figure 3A), whereas an effect of 1.82 ± 0.22 was determined for $V/K_{neomycin\ A}$. No kinetic isotope effects were observed on V for the double mutant AAC(6′)-Iy C109A/C70A using either tobramycin or neomycin C as variable substrate, but inverse isotope effects of 0.74 ± 0.06 and 0.82 ± 0.04 were determined for $V/K_{tobramycin}$ and $V/K_{neomycin\ C}$, respectively (Figure 3B).

Inactivation by Iodoacetamide. AAC(6′)-Iy was found to be inactivated by IAM (Figure 4) as a function of incubation time with IAM. The inactivation consisted of two phases: a rapid, first-order decrease in activity over a 25 min time period, followed by a slower, first-order decrease in activity. The y-intercept of the line fitted to the slower inactivation phase was 1.67 (log of 53% inactivation), suggesting that each phase represented alkylation of approximately half of the total cysteine residues at the active sites. This inactivation

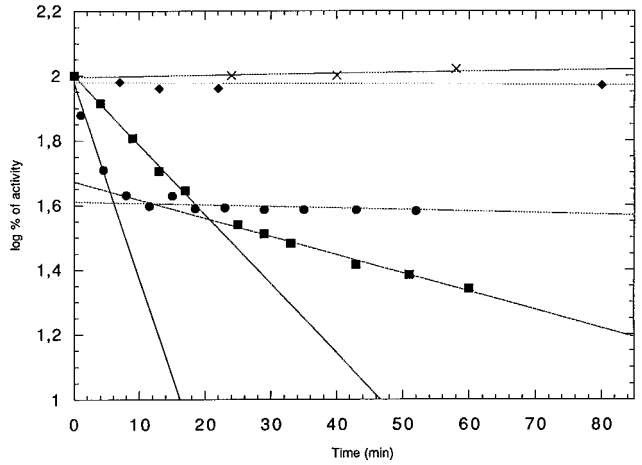


FIGURE 4: Inactivation of AAC(6′)-Iy (■), AAC(6′)-Iy C109A (●), and AAC(6′)-Iy C109A/C70A (◆) by iodoacetamide. Symbols (×) represent the activity of AAC(6′)-Iy incubated in the absence of iodoacetamide.

was completely protected by the addition of 1 mM tobramycin but only very weakly protected by the addition of either 1 mM acetyl-CoA or desulfo-CoA (data not shown). Incubation of the mutant AAC(6′)-Iy C109A enzyme with IAM resulted in a rapid, first-order loss of activity (58% inactivation after 6 min), while the remaining activity was lost only very slowly. The activity of the C109A/C70A double mutant enzyme was unaffected by IAM.

Mass Spectrometry. Analysis of purified AAC(6′)-Iy and mutant AAC(6′)-Iy C109A by electrospray ionization mass spectrometry indicated experimental masses of 17 184 and 17 152 Da, respectively. Analysis of the IAM-inactivated wild-type enzyme revealed that AAC(6′)-Iy was triply carboxamidomethylated with very minor (<5%) amounts of singly and doubly carboxamidomethylated forms of AAC(6′)-Iy observed. These data show that the three cysteine residues in the amino acid sequence of the wild-type enzyme are present as the free thiol and were accessible to IAM. An identical analysis of IAM-inactivated AAC(6′)-Iy C109A revealed that this enzyme was doubly carboxamidomethylated with only very minor (<2%) amounts of singly carboxamidomethylated AAC(6′)-Iy C109A observed.

DISCUSSION

The chromosomally encoded *aac(6′)-Iy* gene was identified from an aminoglycoside-resistant clinical isolate of *S. enterica*, strain BM4362 (*20*). This gene is usually cryptic, and aminoglycoside resistance in BM4362 resulted from a chromosomal deletion that led to its expression by transcriptional fusion. In susceptible *Salmonella* strains, the gene is located downstream from a cluster of seven open reading frames that are homologous to an *E. coli* locus that encodes enzymes putatively involved in carbohydrate transport or metabolism. This genomic environment suggests a normal physiological role for the encoded AAC(6′)-Iy protein similar to that proposed for the AAC(2′)-Ia *O*/*N*-acetyltransferase from *Providencia stuartii* involved in peptidoglycan acetylation (*24*). The AAC(6′)-Iy monomer is a small protein consisting of 145 amino acids that belongs to the major AAC(6′)-I subfamily (*21*). The alignment of the AAC(6′)-Iy sequence with those of other members of this subfamily is shown in Figure 5.

Four regions of conserved sequence observed in all AAC(6′) sequences (motifs A, B, C, D) (*21*) and three others (E, F, G) that are conserved in the major subfamily of which AAC(6′)-Iy is a member can be identified. The majority of the conserved motifs are located in the C-terminal half of these enzymes. We propose that residues in this region are important elements in the active site of the enzyme because of (i) the very high sequence conservation, (ii) previously characterized mutations in this region that affect both acetyltransferase activity and substrate specificity (*21*), and (iii) inspection of the three-dimensional structures of the AAC(3) from *S. marcescens* (*25*) and the *E. faecium* AAC(6′)-Ii (*26*). The CoA binding site of both these *N*-acetyltransferases is composed of a β-α-β motif, with residues in these secondary structural elements making specific interactions with the pantotheine and 3′-phosphomonoester moieties. Pairwise sequence alignments between AAC(3) and AAC(6′)-Iy reveal that essentially all of the critical residues observed in CoA binding are present in residues 80−110 of AAC(6′)-Iy.

Steady-state kinetic parameters were measured for a number of acyl donors and aminoglycosides (Table 1). On the basis of relative *V*/*K* values, acetyl-CoA was the favored acyl donor, while malonyl-CoA and butyryl-CoA exhibited lower aminoglycoside acetyltransferase activity. Propionyl-CoA was inactive as an acyl donor to aminoglycoside substrates but was rapidly hydrolyzed by the enzyme. For acyl-CoA donors, *V* was not substantially different, whereas the K_m values for these substrates were 30−1000 times higher than those observed for acetyl-CoA. The dramatic decrease of *V*/*K* values from the addition of one or two methyl groups on the substrate molecule compared to acetyl-CoA suggests a very sterically restricted acyl donor binding site. These results contrast sharply with studies of acyl donor selectivity determined for the 6′-*N*-acetyltransferases from *E. coli* (*18*) and *E. faecium* (*19*) which use acetyl-CoA, propionyl-CoA, and butyryl-CoA with nearly equivalent efficiencies.

In contrast to its narrow acyl donor specificity, AAC(6′)-Iy has a very broad specificity with respect to aminoglycosides. With the exception of lividomycin A and paromomycin, the only two aminoglycosides that contain a hydroxyl group at the 6′ position, all aminoglycosides tested were substrates. These results demonstrate that the acetyl transfer is regiospecific for the 6′-amino group. Comparisons of steady-state kinetic parameters can be used to probe the substrate specificity of the enzyme. The *V*/*K* values determined for aminoglycosides vary over a 75-fold range, less than that observed for gentamicin acetyltransferase (*15*) but significantly higher than that observed for the 6′-acetyltransferase from *E. faecium* (*19*). Using these kinetic constants and the linear or nonlinear nature of the double reciprocal plots, aminoglycoside substrates can be divided into two classes. The first class includes tobramycin, kanamycin B, kanamycin A, and neomycin A which displayed a relatively low steady-state affinity for the enzyme (K_m values range between 66 and 533 μM) and exhibit linear double reciprocal plots. Neomycin A, or neamine, was the poorest substrate and is the only bicyclic aminoglycoside examined, suggesting that the 3-deoxy-3-aminoglucose substitution at position 6 aids in binding (Figure 1). Kanamycin A differs from kanamycin B at the 2′ position, suggesting that steady-state affinity is enhanced by the 2′-amino group relative to a 2′-

FIGURE 5: Sequence alignment of the AAC(6′) members of the major subfamily. Motifs A, B, C, and D are conserved among the entire AAC(6′) family, while motifs E, F, and G are conserved in the major AAC(6′) subfamily. The predicted acetyl-CoA binding site is indicated, and the cysteines mutagenized in this study are emboldened.

hydroxyl group. Finally, tobramycin differs from kanamycin B in lacking the 3′-hydroxyl group and exhibits a *V*/*K* value four times higher than kanamycin B. The second class includes netilmicin, sisomicin, neomycin C, dibekacin, amikacin, and ribostamycin which exhibited higher steady-state affinity for the enzyme (K_m values range between 8 and 52 μM for the lower K_m value) and biphasic double reciprocal plots referred to as substrate activation (Figure 2). Among these substrates, amikacin, a semisynthetic derivative of kanamycin A, was the poorest substrate. Dibekacin, a 4′-deoxy derivative of tobramycin, is also a relatively poor substrate. Ribostamycin and neomycin C differ from the other aminoglycosides tested in being 4,5-disubstituted as opposed to 4,6-disubstituted deoxystreptamine derivatives. Their substitution pattern on the 6′-deoxy-6′-aminoglucose ring that is the site of modification is identical to neomycin A or kanamycin B. The best substrates of this class are sisomicin and its *N*1-ethyl derivative, netilmicin, which lack the 3′-hydroxyl group and additionally have a Δ4′−5′ unsaturation in the 6′-deoxy-6′-aminoglucose ring.

There is no single structural feature that distinguishes substrates that exhibit linear kinetics from those that do not. All substrates that exhibit linear kinetics possess a 4′-hydroxyl group adjacent to the position of *N*-acetylation. Of the six substrates that yield nonlinear reciprocal plots, three do not contain this group. Of the three that do, two are 4,5-disubstituted aminoglycosides, ribostamycin and neomycin C, while amikacin is modified at *N*1 by the bulky L-α-hydroxybutyramide moiety. Whether these structural differences change the conformation of the 6′-deoxy-6′-aminoglucose ring or prevent the interaction of the 4′-hydroxyl group with the enzyme are uncertain and must await the structural characterization of the enzyme−aminoglycoside complex. As not all aminoglycosides nor acyl-CoA's display substrate activation, the extant asymmetry of monomers in the dimer cannot explain this kinetic behavior. Such aminoglycoside-dependent shifts from linear to nonlinear kinetics have been observed for gentamicin acetyltransferase (*16*) and proposed to be the result of changes in the kinetic mechanism. We therefore performed studies to determine the kinetic mechanism.

The intersecting initial velocity pattern obtained with tobramycin and acetyl-CoA (Supporting Information) argues against a ping-pong mechanism and suggests a sequential kinetic mechanism where both substrates must be bound at the active site for catalysis to occur. To distinguish between the ordered or random addition of substrates, two methods were used. Dead-end inhibitors are powerful tools that can be used to identify the ordered addition of substrates. Desulfo-CoA displayed linear, competitive inhibition versus acetyl-CoA and linear, noncompetitive inhibition versus tobramycin (Table 2). These data are compatible with the ordered addition of acetyl-CoA followed by tobramycin. Lividomycin, which possesses a 6′-hydroxyl group and is not a substrate for AAC(6′)-Iy, is a potent inhibitor and exhibits linear, noncompetitive inhibition versus both acetyl-CoA and tobramycin. These results suggest that lividomycin can bind to the free enzyme E−acetyl-CoA complex and probably also the E−CoASH product complex and argue for the random binding of substrates to the enzyme. A second

kinetic diagnostic that has been used to discriminate between various kinetic mechanisms is the determination of the dependence of V and V/K for one substrate not on the concentration but rather on the identity of the second substrate. This method was, in fact, used to determine the kinetic mechanism of kanamycin 6′-*N*-acetyltransferase to be rapid equilibrium random (*17*). We determined the V/K value for acetyl-CoA at saturating concentrations of four aminoglycoside substrates. For either a ping-pong or an ordered mechanism in which acetyl-CoA binds to the free enzyme, the V/K value for acetyl-CoA should be independent of aminoglycoside substrate identity, since $V/K_{\text{acetyl-CoA}}$ may be considered to be the pseudo-first-order rate constant for nucleotide binding to the free enzyme. The determined values of $V/K_{\text{acetyl-CoA}}$ depend on the identity of the aminoglycoside used, thus arguing either for the initial ordered binding of aminoglycoside followed by acetyl-CoA (our dead-end inhibition data, however, argue against this) or for the random binding of substrates. The dependence of $V/K_{\text{acetyl-CoA}}$ on aminoglycoside identity exactly parallels the dependence on V, arguing that acetyl-CoA steady-state affinity is unaffected by the nature of the aminoglycoside substrate. Our conclusion that the enzyme uses a random kinetic mechanism is also supported by the observation of a very slow, aminoglycoside-independent hydrolysis of acetyl-CoA, the rapid, aminoglycoside-independent hydrolysis of propionyl-CoA, and the ability of tobramycin to prevent the alkylation of residues at the active site (see below).

Two limiting cases of random kinetic mechanism exist: steady-state random mechanisms where substrate binding steps, product release steps, or chemistry can be partially rate-limiting and the rapid equilibrium random mechanism where all substrate binding and product release steps are fast and chemistry is rate-limiting. In the latter case, isotopic probes might provide useful tools, especially the examination of solvent kinetic isotope effects.

Solvent kinetic isotope effects were determined at pH 7.5, a region where the kinetic parameters are independent of small changes in pH(D) (data not shown). To simplify the analysis, we chose two substrates that exhibited linear double reciprocal plots, tobramycin and neomycin A. Solvent kinetic isotope effects, determined at a saturating concentration of acetyl-CoA and varying concentrations of tobramycin, were normal and yielded fitted values of $^{D_2O}V = 1.28 \pm 0.03$ and $^{D_2O}V/K = 1.01 \pm 0.08$. This same analysis using neomycin A as substrate yielded fitted values of $^{D_2O}V = 1.27 \pm 0.03$ and $^{D_2O}V/K = 1.81 \pm 0.23$. These values are extrapolated to 100% D_2O and assume a linear dependence of the kinetic parameters on solvent isotopic composition. The lack of an effect of solvent isotopic composition on $V/K_{\text{tobramycin}}$ suggests that tobramycin is "sticky" and partitions through catalysis faster than it dissociates. Neomycin A was the poorest substrate, with a relative V/K value 75 times lower than tobramycin. The solvent kinetic isotope effect of 1.8 reflects the lower commitment to catalysis for this substrate. The solvent kinetic isotope effects on V were small and equivalent for the two substrates. Although small, the inequality of the ^{D_2O}V and $^{D_2O}V/K$ values argues against a rapid equilibrium random kinetic mechanism for AAC(6′)-Iy, where chemistry is the sole rate-limiting step.

Incubation of AAC(6′)-Iy with 5 mM IAM resulted in a biphasic, time-dependent inactivation of tobramycin acetyltransferase activity. Extrapolation of the slower phase to zero time corresponded to inactivation of approximately half of the total activity. The alkylation of two differently accessible or reactive cysteine residues or the alkylation of one residue affecting the reactivity or accessibility of a second can explain this biphasic inactivation, which could be prevented by the inclusion of tobramycin in the preincubation mixture. AAC(6′)-Iy contains three cysteine residues at positions 70, 109, and 145. Cysteine 109 is conserved in all members of the major 6′-*N*-acetyltransferase family, while the residue corresponding to position 70 in AAC(6′)-Iy is either a cysteine or a threonine. Cysteine 145 is the terminal residue of AAC(6′)-Iy and does not align with other polar residues (Figure 5). Mass spectrometric data indicated that all three cysteine residues were carboxamidomethylated in the WT enzyme (Supporting Information). To investigate the function of cysteine residues, the C109A and double C109A/C70A mutants were prepared. Both of these enzymes were active and exhibited an enzyme concentration-dependent tobramycin *N*-acetyltransferase activity, indicating that neither the C109 nor C70 residues were functioning as critical acid/base residues in the reaction. AAC(6′)-Iy C109A/C70A was not inactivated by IAM, indicating that alkylation of cysteine 145 was not related to AAC(6′)-Iy inactivation by IAM. However, IAM inactivation of AAC(6′)-Iy C109A remained biphasic, with the first phase occurring more rapidly and the second phase more slowly than observed with the WT enzyme. Mass spectrometric data revealed the carboxamidomethylation of both remaining cysteine residues in the C109A mutant. These data argue against the biphasic inactivation data observed for the WT enzyme as being due to differential reactivity of two cysteines (C109 and C70) and rather must be attributed to an intrinsic asymmetry of the enzyme. The inactivation of both the WT and C109A mutant presumably results from the alkylation of C70, which prevents subsequent substrate binding either by steric hindrance or by preventing essential enzyme−substrate interactions. Cysteines 109 and 70 thus may be located in or near the active site and likely function in enzyme−aminoglycoside interactions.

To complement these physiochemical data, we kinetically characterized the substrate specificity of the single and double mutants. The steady-state K_m value for acetyl-CoA was very slightly elevated in the C109A mutant (17 vs 10 μM for WT); however, the K_m values for aminoglycoside substrates were either slightly elevated (e.g., amikacin) or lowered. The maximum velocities for all substrates were significantly higher, resulting in V/K values for the C109A mutant enzyme that were greater than those for the WT enzyme. The substrate selectivity, as measured by V/K values, changed dramatically, with kanamycin B exhibiting the highest activity. Most surprisingly, those substrates that exhibited linear reciprocal plots with the WT enzyme exhibited nonlinear, concave-downward reciprocal plots with the C109A mutant and vice versa. This inversion of both kinetic behavior and substrate selectivity is extremely unusual and suggests a role for C109 in substrate recognition.

The kinetic parameters exhibited by the double C109A/C70A mutant are equally intriguing. The K_m value for acetyl-CoA was elevated 8-fold (77 vs 10 μM for WT), while the K_m values for aminoglycosides were elevated 10- to 50-fold compared to the WT or C109A enzymes. The presence of

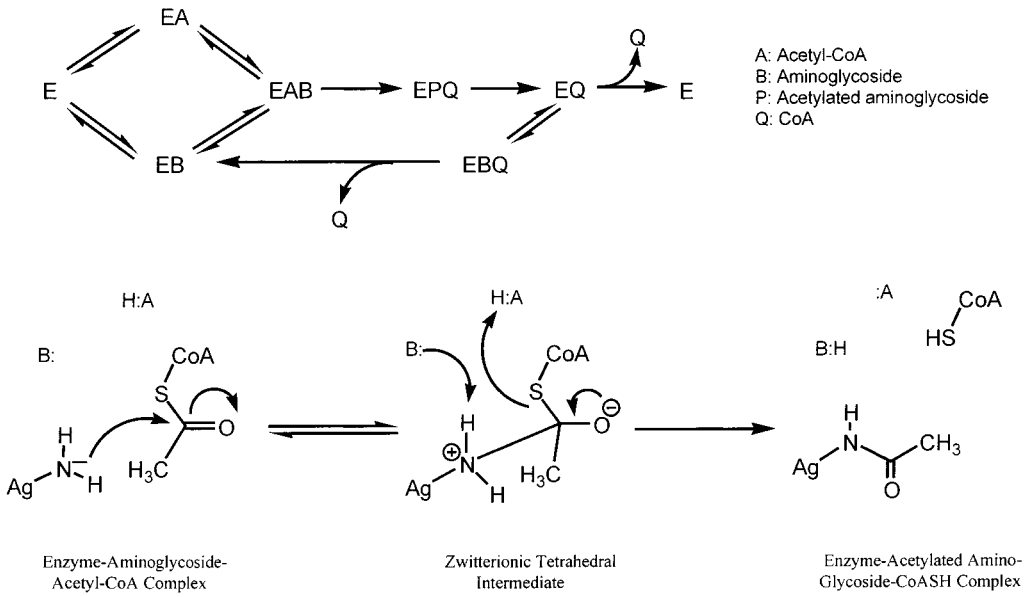


FIGURE 6: Proposed kinetic and chemical mechanisms for aminoglycoside (Ag) acetylation by AAC(6′)-Iy.

the second mutation abolished all nonlinear reciprocal kinetic behavior for the four substrates examined but had only a modest effect on the catalytic turnover. These data support our previous conclusion that neither C109 nor C70 is involved in the chemical reaction, but rather in substrate binding and kinetic behavior, and suggest that substrate binding has been severely affected in the double mutant. We suspected that these mutationally induced changes would affect the relative rate-limiting nature of substrate binding/product dissociation or catalytic steps and that these changes would be observed in the solvent isotope dependence of the reaction catalyzed by the mutant enzymes. In fact, using the C109A/C70A double mutant and either tobramycin or neomycin C, solvent deuterium kinetic isotope effects of 1.0 were observed on *V*, while inverse effects of 0.7−0.8 were observed on *V*/*K*. *V*/*K* for substrate includes rate constants for all steps between the binding of the variable substrate, aminoglycosides in this case, and the first irreversible step, usually considered as the release of the first product. Given the strongly exothermic nature of the conversion of a thioester (acetyl-CoA) to an amide (acetyl-aminoglycoside), the breakdown of the tetrahedral intermediate may be irreversible. Some step in this sequence, or a conformational change accompanying this step, may be responsible for the inverse solvent kinetic isotope effect on *V*/*K*. This would be followed by the slow release of products, in particular CoASH, which would be independent of solvent isotopic composition yielding a ^{D_2O}V of 1.0.

Taken together, these data allow us to propose a model for the AAC(6′)-Iy-catalyzed reaction (Figure 6). The sequential nature of the kinetic mechanism that has been observed for all aminoglycoside *N*-acetyltransferases studied to date (*16*, *17*) requires that both substrates be bound at the active site before chemistry can occur. Substrate binding is proposed to occur randomly with little synergism between acetyl-CoA and aminoglycoside binding. Although products may also be released randomly, we show the ordered release of acetylated aminoglycoside followed by CoA (upper panel). The binding of aminoglycoside, B, to the EQ complex and the more rapid dissociation of CoA from the EBQ complex, compared to the EQ complex, are sufficient to account for the substrate activation that we observe with most aminoglycosides.[2] A second explanation invokes cooperativity between the monomers of the dimeric enzyme. In this case, binding of specific aminoglycosides to one subunit results in a considerably decreased affinity of the other subunit for substrate. The kinetic behavior of the mutant enzymes could be explained by changes in specific monomer−monomer interactions involved in this cooperativity for AAC(6′)-Iy C109A and by the complete abolition of such interactions, or dimer dissociation, for AAC(6′)-Iy C109A/C70A. We show the chemical reaction being initiated by the attack of the unprotonated 6′-amino group on the carbonyl group of acetyl-CoA. The zwitterionic tetrahedral intermediate collapses, with possible general base assistance, to generate the acetylated aminoglycoside and the thiol, after proton transfer from a general acid. This hypothesis is also supported by the solvent kinetic isotope effect data for the WT and the double mutant.

A major unanswered question of the present study is the physiological role of the *S. enterica aac(6′)-Iy*-encoded 6′-*N*-acetyltransferase. It is very unlikely that this "cryptic" enzyme functions as an antibiotic-modifying enzyme normally. The gene is located after a cluster of open reading frames that are homologous to genes identified in *E. coli* that may represent a sugar transporter and metabolizing system. Since we have been unable to demonstrate *O*-acetylation of aminoglycosides, in contrast to AAC(2′)-Ia from *P. stuartii* (*24*), the chromosomally encoded AAC(6′)-Iy may not be involved in sugar *O*-acetylation. Very recently, the chromosomally encoded AAC(6′)-Ii of *E. faecium* has been shown to catalyze acetyl transfer from acetyl-CoA to proteins (*26*), as has the GCN5-like aminoglycoside 3-*N*-acetyltransferase (*25*). Even if AAC(6′)-Iy can perform this function, it will be difficult to identify its normal, physiological protein substrate.

[2] The rate equation for this mechanism has been derived and used to fit the data in Figure 2. The derivation is included in Supporting Information.

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SUPPORTING INFORMATION AVAILABLE

Derivation of the rate equation for the kinetic mechanism, two figures showing initial velocity patterns for tobramycin N-acetylation and alternate substrate kinetics, and one table giving mass spectrometric data for iodoacetamide alkylation. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Davis, B. D. (1987) *Microbiol. Rev. 51*, 341−350.
2. Hooper, I. R., Ito, I., Koeda, T., Kondo, S., Mitsuhashi, S., Okuda, T., Tanaka, T., Tsuchiya, K., Umemura, H., Umezawa, H., Umezawa, S., and Yokota, M. (1982) in *Handbook of Experimental Pharmacology* (Umezawa, H., and Hooper, I. R., Eds.) Springer-Verlag, Berlin, Heidelberg, and New York.
3. Dang, P., Gutmann, L., Quentin, C., Williamson, R., and Collatz, E. (1988*) Rev. Infect. Dis. 10*, 899−904.
4. Taber, H. W., Mueller, J. P., Miller, P. F., and Arrow, A. S. (1987) *Microbiol. Rev. 51*, 439−457.
5. Kashiwagi, K., Tsuhako, M. H., Sakata, K., Saisho, T., Igarashi, A., da Costa, S. O., and Igarashi, K. (1998) *J. Bacteriol. 180*, 5484−5488.
6. Moore, R. A., Deshazer, D., Reckseidler, S., Weissman, A., and Woods, D. E. (1999) *Antimicrob. Agents Chemother. 43*, 465−470.
7. Ramos Aires, J., Köhler, T., Nikaido, H., and Plésiat, P. (1999*) Antimicrob. Agents Chemother. 43*, 2624−2628.
8. Miller, G. H., Sabatelli, F. J., Naples, L., Hare, R. S., and Shaw, K. J. (1995*) J. Chemother. 7* (Suppl. 2), 31−44.
9. Wright, G. D. (1999) *Curr. Opin. Microbiol. 2*, 499−503.
10. Courvalin, P., Carlier, C., and Collatz, E. (1980) *J. Bacteriol. 143*, 541−551.
11. Rather, P. N., Orosz, E., Hare, R. S., Miller, G., and Shaw, K. J. (1993) *J. Bacteriol. 175*, 6492−6498.
12. Shaw, K. J., Rather, P. N., Sabatelli, F., Mann, P., Munayyer, H., Mierzwa, R., Petrikkos, G., Hare, R. S., Miller, G. H., Bennett, P., and Downey, P. (1992*) Antimicrob. Agents Chemother. 36*, 1447−1455.
13. Rudant, E., Bouvet, P., Courvalin, P., and Lambert, T. (1999) *System. Appl. Microbiol. 22*, 59−67.
14. Rather, P. N., Munayyer, H., Mann, P. A., Hare, R. S., Miller, G. H., and Shaw, K. J. (1992) *J. Bacteriol. 174*, 3196−3203.
15. Williams, J. W., and Northrop, D. B. (1978) *J. Biol. Chem. 253*, 5908−5914.
16. Williams, J. W., and Northrop, D. B. (1978) *J. Biol. Chem. 253*, 5902−5907.
17. Radika, K., and Northrop, D. B. (1984) *J. Biol. Chem. 259*, 12543−12546.
18. Radika, K., and Northrop, D. B. (1984) *Biochemistry 23*, 5118−5122.
19. Wright, G. D., and Ladak, P. (1997) *Antimicrob. Agents Chemother. 41*, 956−960.
20. Magnet, S., Courvalin, P., and Lambert, T. (1999) *J. Bacteriol. 181*, 6650−6655.
21. Shaw, K. J., Rather, P. N., Hare, R. S., and Miller, G. H. (1993) *Microbiol. Rev. 57*, 138−163.
22. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
23. Cleland, W. W. (1979) *Methods Enzymol. 78*, 103−188.
24. Payie, K. G., and Clarke, A. J. (1997) *J. Bacteriol. 179*, 4106−4114.
25. Wolf, E., Vassilev, A., Makino, Y., Sali, A., Nakatani, Y., and Burley, S. K. (1998) *Cell 94*, 439−449.
26. Wybenga-groot, L., Draker, K. A., Wright, G. D., and Berghuis, A. M. (1999) *Structure 7*, 497−507.

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