The Molecular Basis of the Expansive Substrate Specificity of the Antibiotic Resistance Enzyme Aminoglycoside Acetyltransferase-6'-Aminoglycoside Phosphotransferase-2"* 

THE ROLE OF ASP-99 AS AN ACTIVE SITE BASE IMPORTANT FOR ACETYL TRANSFER* 

Received for publication, November 15, 2002, and in revised form, January 31, 2003 Published, JBC Papers in Press, February 3, 2003, DOI 10.1074/jbc.M211680200

David D. Boehr, Stephen I. Jenkins, and Gerard D. Wright‡ 

From the Antimicrobial Research Centre, Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

The most frequent determinant of aminoglycoside antibiotic resistance in Gram-positive bacterial pathogens is a bifunctional enzyme, aminoglycoside acetyltransferase-6'-aminoglycoside phosphotransferase-2 (AAC(6')-aminoglycoside phosphotransferase-2', capable of modifying a wide selection of clinically relevant antibiotics through its acetyltransferase and kinase activities. The aminoglycoside acetyltransferase domain of the enzyme, AAC(6')-Ie, is the only member of the large AAC(6') subclass known to modify fortimicin A and catalyze O-acetylation. We have demonstrated through solvent isotope, pH, and site-directed mutagenesis effects that Asp-99 is responsible for the distinct abilities of AAC(6')-Ie. Moreover, we have demonstrated that small planar molecules such as 1-(bromomethyl)phenanthrene can inactive the enzyme through covalent modification of this residue. Thus, Asp-99 acts as an active site base in the molecular mechanism of AAC(6')-Ie. The prominent role of this residue in aminoglycoside modification can be exploited as an anchoring site for the development of compounds capable of reversing antibiotic resistance in vivo.

Clinical usage of aminoglycoside-aminocyclitol antibiotics is blocked by the presence of aminoglycoside modifying enzymes (AMEs)‡ in resistant organisms (for review see Refs. 1 and 2). Bacteria become protected from aminoglycosides, because the modified antibiotics can no longer bind with high affinity to their target, the A-site of the small ribosomal subunit, because of unfavorable steric and/or electrostatic constraints (3). The AMEs are a diverse set of proteins composed of three families: aminoglycoside nucleotidyltransferases, aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs).

The most clinically important AME in Gram-positive bacte-

"This work was supported by the Canadian Institutes of Health Research Grant MT-13536 and by a Canada Research Chair in Antimicrobial Biochemistry (to G. D. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 905-525-9140 (ext. 22454); Fax: 905-525-9033; E-mail: wrightge@mcmaster.ca.

* This work was supported by the Canadian Institutes of Health Research Grant MT-13536 and by a Canada Research Chair in Antimicrobial Biochemistry (to G. D. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 905-525-9140 (ext. 22454); Fax: 905-525-9033; E-mail: wrightge@mcmaster.ca.

The most frequent determinant of aminoglycoside antibiotic resistance in Gram-positive bacterial pathogens is a bifunctional enzyme, aminoglycoside acetyltransferase-6'-aminoglycoside phosphotransferase-2 (AAC(6')-aminoglycoside phosphotransferase-2', capable of modifying a wide selection of clinically relevant antibiotics through its acetyltransferase and kinase activities. The aminoglycoside acetyltransferase domain of the enzyme, AAC(6')-Ie, is the only member of the large AAC(6') subclass known to modify fortimicin A and catalyze O-acetylation. We have demonstrated through solvent isotope, pH, and site-directed mutagenesis effects that Asp-99 is responsible for the distinct abilities of AAC(6')-Ie. Moreover, we have demonstrated that small planar molecules such as 1-(bromomethyl)phenanthrene can inactive the enzyme through covalent modification of this residue. Thus, Asp-99 acts as an active site base in the molecular mechanism of AAC(6')-Ie. The prominent role of this residue in aminoglycoside modification can be exploited as an anchoring site for the development of compounds capable of reversing antibiotic resistance in vivo.

Clinical usage of aminoglycoside-aminocyclitol antibiotics is blocked by the presence of aminoglycoside modifying enzymes (AMEs)‡ in resistant organisms (for review see Refs. 1 and 2). Bacteria become protected from aminoglycosides, because the modified antibiotics can no longer bind with high affinity to their target, the A-site of the small ribosomal subunit, because of unfavorable steric and/or electrostatic constraints (3). The AMEs are a diverse set of proteins composed of three families: aminoglycoside nucleotidyltransferases, aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs).

The most clinically important AME in Gram-positive bacte-

"This work was supported by the Canadian Institutes of Health Research Grant MT-13536 and by a Canada Research Chair in Antimicrobial Biochemistry (to G. D. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 905-525-9140 (ext. 22454); Fax: 905-525-9033; E-mail: wrightge@mcmaster.ca.

The most frequent determinant of aminoglycoside antibiotic resistance in Gram-positive bacterial pathogens is a bifunctional enzyme, aminoglycoside acetyltransferase-6'-aminoglycoside phosphotransferase-2 (AAC(6')-aminoglycoside phosphotransferase-2', capable of modifying a wide selection of clinically relevant antibiotics through its acetyltransferase and kinase activities. The aminoglycoside acetyltransferase domain of the enzyme, AAC(6')-Ie, is the only member of the large AAC(6') subclass known to modify fortimicin A and catalyze O-acetylation. We have demonstrated through solvent isotope, pH, and site-directed mutagenesis effects that Asp-99 is responsible for the distinct abilities of AAC(6')-Ie. Moreover, we have demonstrated that small planar molecules such as 1-(bromomethyl)phenanthrene can inactive the enzyme through covalent modification of this residue. Thus, Asp-99 acts as an active site base in the molecular mechanism of AAC(6')-Ie. The prominent role of this residue in aminoglycoside modification can be exploited as an anchoring site for the development of compounds capable of reversing antibiotic resistance in vivo.

Clinical usage of aminoglycoside-aminocyclitol antibiotics is blocked by the presence of aminoglycoside modifying enzymes (AMEs)‡ in resistant organisms (for review see Refs. 1 and 2). Bacteria become protected from aminoglycosides, because the modified antibiotics can no longer bind with high affinity to their target, the A-site of the small ribosomal subunit, because of unfavorable steric and/or electrostatic constraints (3). The AMEs are a diverse set of proteins composed of three families: aminoglycoside nucleotidyltransferases, aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs).

The most clinically important AME in Gram-positive bacte-

"This work was supported by the Canadian Institutes of Health Research Grant MT-13536 and by a Canada Research Chair in Antimicrobial Biochemistry (to G. D. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 905-525-9140 (ext. 22454); Fax: 905-525-9033; E-mail: wrightge@mcmaster.ca.
Active Site Base in Aminoglycoside Acetyltransferase

acetyltransferase (also known as aminoglycoside N-acetyltransferase or AANAT) bound with a bisubstrate analog (25–28), further argues for a direct acetyl transfer mechanism. For such a transfer to occur, the amine of the acceptor substrate must react with the carbonyl carbon of acetyl-CoA. However, at physiological pH, amines tend to be fully protonated and, hence, are not in a chemically competent state to accept the acetyl group. Consequently, an active site base has been proposed to deprotonate the amine to generate a more potent nucleophile capable of being acetylated.

Structural and functional analyses of AANAT (25, 26, 29) and tGCN5 (24, 30, 31) have implicated His-122 and Glu-173, respectively, as the catalytic base in these enzymes, and mutation of Glu-173 to Gln results in an active site base.

In contrast to either tGCN5 or AANAT, glucosamine 6-phosphate N-acetyltransferase (GNA1) appears to lack an active site base since mutational analysis of Glu-173 to Gln results in a ~1000-fold decrease in GCN5 acetyltransferase activity (30, 31). However, studies with AANAT have been less enlightening, as mutation of His-122 to Gln only results in a 6-fold decrease in activity (29). It has been suggested that substrate deprotonation does not occur directly, but through a series of well-ordered water molecules, or “proton wire,” connecting the substrate amine and the catalytic base (reviewed in Refs. 17). In such a scenario, other residues in the active site that are capable of accepting hydrogen from these water molecules may compensate in part for the loss of the normal proton acceptor. Indeed, His-120 in AANAT appears to play this role and only when both His-122 and His-120 are mutated to Gln is there a much more substantial effect consistent with base catalysis (29).

In contrast to either tGCN5 or AANAT, glucosamine 6-phosphate N-acetyltransferase (GNA1) appears to lack an active site base since mutational analysis of Glu-173 to Gln results in a ~1000-fold decrease in GCN5 acetyltransferase activity (30, 31). However, studies with AANAT have been less enlightening, as mutation of His-122 to Gln only results in a 6-fold decrease in activity (29). It has been suggested that substrate deprotonation does not occur directly, but through a series of well-ordered water molecules, or “proton wire,” connecting the substrate amine and the catalytic base (reviewed in Refs. 17). In such a scenario, other residues in the active site that are capable of accepting hydrogen from these water molecules may compensate in part for the loss of the normal proton acceptor. Indeed, His-120 in AANAT appears to play this role and only when both His-122 and His-120 are mutated to Gln is there a much more substantial effect consistent with base catalysis (29).

Here we have addressed the role of an active base in the catalytic mechanism of AAC(6’)-Ie. We demonstrate through solvent isotope effects, pH studies, and mutational analysis that Asp-99 fulfills the role of an active site base in AAC(6’)-Ie to catalyze O-acetyl transfer. Although Asp-99 provides the enzyme with an enhanced detoxification profile, we further demonstrate that compounds such as 1-(bromomethyl)phenanthrene can inactivate AAC(6’)-Ie by covalently modifying this residue. These compounds have the potential to be further developed as potent inhibitors of AAC(6’)-Ie's substrate to overcome aminoglycoside resistance in vivo.

EXPERIMENTAL PROCEDURES

Reagents—Kanamycin A was from Bioshop (Burlington, Ontario, Canada). 4,4’-Dithiodipyrindine was from Amsbera Biosciences (Baie d’Urfé, Quebec, Canada). All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. All oligonucleotide primers were synthesized at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. The purification of N-terminal hexahistidine-tagged AAC(6’)-APH(2’)(25) has been previously described (15), and mutant enzymes were purified similarly. AAC(6’)-Ie Kinetic Assays—Aminoglycoside acetylation by AAC(6’)-Ie was normally monitored by the in situ titration of free coenzyme A product with 4,4’-dithiodipyrindine as previously described (8). However, assays were scaled down from 1-m1 to 250-µ1 volumes, so they could be conducted in 96-well microtiter plates using a Molecular Devices SpectraMax Plus microtiter plate reader. For more sensitive assays, acetyltransferase activity was determined using [1-14C]acetyl-CoA and a phosphocellulose binding assay described previously (16). Reaction mixtures typically contained 25 mM HEPES-NaOH, pH 7.5, 0.1 µCi of [1-14C]AcCoA (200 µCi final concentration), 1 mM aminoglycoside substrate, and 0.3 nmol of pure AAC(6’)-Ie, and were allowed to proceed for 10 min at 37°C.

Initial rates were fit to the Michaelis-Menten equation (1) using Graphit 4.0 (34), where \( v = \frac{(k_{cat}E)}{K_m + [S]} \) (Eq. 1)

\[ v = \frac{k_{cat}E}{K_m + [S]} \]  

where \( v \) is the initial velocity, \( E \) is the total amount of enzyme in the assay and \( [S] \) is the concentration of substrate. The concentrations of acetyl-CoA and kanamycin A were held at 900 µM when measuring the steady-state kinetic parameters for aminoglycoside substrate and acetyl-CoA, respectively.

Solvent Viscosity, Solvent Isotope, and pH Effects for AAC(6’)-Ie—The solvent viscosity, solvent isotope, and pH effects for AAC(6’)-Ie were determined by varying the buffer conditions of the standard assay. For the solvent viscosity effect experiments, steady-state kinetic parameters were determined with varying concentrations of the microviscosogen glycerol (0, 15, 22.5, and 30% v/v). The viscosity of the solutions was determined using an Ostwald viscometer in triplicate, and the slope of a plot of relative viscosity versus rate / rate viscosity reveals the solvent viscosity effect (SVE). Steady-state kinetic parameters were also performed in the macroviscosogen polyethylene glycol 8000 (6.7% w/v) and were not found to have a significant effect on the rate constants.

Solvent isotope effects were measured by conducting kinetic analyses in D2O (99.9% from Isotec), where the final amount of H2O was not more than 5%. pH values were determined by measuring pH and adding 0.4 unit (pD = pH + 0.4). The proton inventory study was conducted by adding ratios of buffer in H2O and D2O and making appropriate corrections for the addition of enzyme and substrates to calculate the final amount of D2O in the enzyme assay solution. Buffers with overlapping pH ranges were used to investigate the effect of pH on enzyme activity. Buffers used were: 25 mM MOPS-NaOH (pH 6.0–6.5), 25 mM MES-NaOH (pH 6.5–7.5), 25 mM HEPES-NaOH (pH 7.0–8.0), and 25 mM glycine (pH 8.0–9.5). None of the buffers gave any significant nonspecific effects. The data were analyzed using Graphit 4.0 (34). For AAC(6’)-Ie WT, which displays two ionizations, the data can be fit to the following equation through nonlinear regression,

\[ \log v = \log C + \log \left(1 + \frac{K_m}{K_a} \right) \]  

where \( v \) is the first-order (\( k_{cat} \)) or second-order (\( k_{cat}/K_m \)) rate constant, \( K_a \) and \( K_m \) are the acid and base equilibrium constants, respectively, \( C \) is the pH-independent value, and \( H \) is the proton concentration.

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange method (Stratagene, La Jolla, CA). The appropriate mutagenic primers (Tyr-86 → Phe, GAGATGCTGCTATGGTATGGAACAATTTATAGGAGAGCC; Asp-99 → Ala, GAGATGCTGCTATGGTATGGAACAATTTATAGGAGAGCC; Asp-99 → Asn, GAGATGCTGCTGCTATGGTATGGAACAATTTATAGGAGAGCC; and Asp-99 → Glu, GAGATGCTGCTGCTATGGTATGGAACAATTTATAGGAGAGCC) and their reverse complements were used in combination with 20 ng of template DNA (pET15AACAPH (15)) in an in vitro DNA polymerase (Stratagene, La Jolla, CA)–catalyzed PCR reactions. After parental DNA was digested with DpnI, mutant plasmid DNA was transformed into CaCl2–competent Escherichia coli XL-1 Blue. Positive clones were sequenced in their entirety and then used to transform into E. coli BL21(DE3) for subsequent protein purification.

Minimum Inhibitory Concentration Determinations with Fortimicin A—Minimum inhibitory concentration (MIC) determinations were performed as described in Ref. 35, where the MICs for E. coli BL21(DE3) carrying control plasmid pET15b(+) were compared with E. coli BL21(DE3) carrying pET15AACCAPH and appropriate plasmids with site mutants in the aac(6’)-aph(2’)(25) gene.
**Synthesis of 1-(Bromomethyl)phenanthrene, 1-(Bromomethyl)naphthalene, and 9-(Chloromethyl)phenanthrene—** The preparation of 1-(bromomethyl)phenanthrene and 9-(chloromethyl)phenanthrene by using N-bromo- and N-chlorosuccinimide, respectively, has been previously reported (36). 1-(Bromomethyl)naphthalene was prepared in an analogous manner using N-bromosuccinimide: thin-layer chromatography, Rf 0.42. 1H NMR (200 MHz, CDCl3): δ 8.57 (d, J = 8.57 Hz, 1H, CH), 7.90 (t, J = 9.18 Hz, 2H, CH), 7.43—7.67 (m, 4 H, CH), 4.99 (s, 2H, CH2Br). 13C NMR (50 MHz, CDCl3): δ 138.9, 133.2, 131.0, 129.7, 128.8, 127.7, 126.5, 126.1, 125.3, 123.6, and 31.7.

**Inactivation of AAC(6′)-Ie by (Halomethyl)phenanthrenes and (Halomethyl)naphthalenes—** Inactivation experiments were carried out by incubating enzyme (0.2—2 μM) with compound (50—200 μM) dissolved in Me2SO (0.04% v/v total) in 240 mM HEPES-NaOH (pH 7.5), and 2 mM 4,4′-dithiodipyridine at 37°C for 10—40 min, prior to the addition of kanamycin A and acetyl-CoA (300 μM final concentrations) to measure acetyltransferase activity. For the substrate protection experiments, kanamycin A (500 μM) and/or desulfo-CoA (100 μM) was incubated with the inactivation mixture for 10 min before the addition of acetyl-CoA (500 μM final). The concentration of desulfo-CoA used in these experiments (100 μM) was in the range of the IC50 for desulfo-CoA using 300 μM kanamycin A and 300 μM acetyl-CoA. The effect of pH on inactivation was determined by conducting inactivation experiments in the following buffers: 25 mM MOPS-NaOH (pH 6.5), 25 mM HEPES-NaOH (pH 7.0—8.0), and 25 mM glycyglycine (pH 8.5).

**RESULTS AND DISCUSSION**

**AAC(6′)-Ie Can Acetylate Aminoglycoside 6′-OH and 6′-NH2—** AAC(6′)-Ie is unique in that it is the only known member of the AAC(6′) subclass that can acetylate fortimicin A, and it also has the ability to O-acetylate aminoglycosides at the 6′ position as represented by paromomycin (8) (Fig. 1 and Table I). The pKa of the 6′-NH2 of aminoglycosides related to neamine (37), and kanamycin A (38) has previously been determined to be 8.6. As such, the significant proportion of the aminoglycoside will be in the fully protonated state at physiological pH, and a catalytic base would be predicted to increase the efficiency of the reaction, although there is no absolute requirement for an active site base. An active site base would be predicted to be more critical with 6′-OH aminoglycosides, because the pK of the hydroxyl will be in the range of 14—16.

**Solvent Viscosity and Solvent Isotope Effects for AAC(6′)-Ie—**

AAC(6′)-Ie has been shown to follow a random order Bi Bi kinetic mechanism (23). To further define the kinetic mechanism, we performed solvent viscosity effect (SVE) experiments with glycerol as the microviscosogen to identify the rate-determining step(s) in the catalytic cycle. A significant SVE indicates that one or more rate-determining steps are diffusion-controlled, with either substrate coming to the enzyme, product leaving the enzyme, or a diffusion-controlled conformational change, whereas the lack of a significant SVE effect indicates a diffusion-independent rate-determining step, such as a viscosity-independent conformational change or chemistry at the active site.

There was a minor solvent viscosity effect for both kcat and kcat/Km with acetyl-CoA as the variable substrate, but there was not a significant SVE for either kcat or kcat/Km with kanamycin A as the variable substrate (Table II). The lack of a SVE is consistent with the chemical step(s) being rate-limiting.

The importance of proton extraction in the chemical step(s) was investigated by determining solvent isotope effects (SIEs) (kcat/kcat). The only significant SIE determined for AAC(6′)-Ie was for the second order rate constant (kcat/Km) with respect to kanamycin A (Table II). As solvent isotope effects may be related to one or multiple exchangeable hydrogens, we further defined the solvent isotope effect for kanamycin A by performing a proton inventory study, where the steady-state kinetic parameters are determined in varying ratios of H2O to D2O (Fig. 2). The linearity of the curve is consistent with the SIE being due to mobilization of only one hydrogen (39), likely a hydrogen extracted from the 6′-NH2 position of kanamycin A. Thus, these results are consistent with deprotonation of kanamycin A being important in productive aminoglycoside capture.

**pH Effects for AAC(6′)-Ie—** The effects of pH on steady-state kinetic parameters can give insight into the required ionization state of important enzyme active site residues. The pH effects for AAC(6′)-Ie showed two important ionizations whether acetyl-CoA or neamine was the variable substrate (Fig. 3, A and B, and Table III). These pK values may be related to the required ionization states of enzymatically important residues. Thus, for example, the acidic limb of the pH curve (pK...
Active Site Base in Aminoglycoside Acetyltransferase

Steady-state kinetic parameters of wild type and mutant AAC(6\textsuperscript{r})-Ie

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ ($\mu M$)</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$k_{cat}/K_m$ ($M^{-1}s^{-1}$)</th>
<th>$k_{cat}^{WT}/k_{cat}^{MUT}$</th>
<th>$k_{cat}^{WT}/K_m^{WT}/k_{cat}^{MUT}/K_m^{MUT}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAC(6\textsuperscript{r})-Ie wild type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA$^{a}$</td>
<td>38 ± 5</td>
<td>1.2 ± 0.3</td>
<td>$3.1 \times 10^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanamycin A$^{a}$</td>
<td>31 ± 3</td>
<td>1.7 ± 0.2</td>
<td>$5.6 \times 10^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neamine</td>
<td>78.3 ± 15.7</td>
<td>0.43 ± 0.02</td>
<td>$5.6 \times 10^2$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fortimicin A</td>
<td>25.4 ± 5.5</td>
<td>0.28 ± 0.04</td>
<td>$1.1 \times 10^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paromomycin</td>
<td>324 ± 45</td>
<td>0.059 ± 0.003</td>
<td>$1.8 \times 10^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAC(6\textsuperscript{r})-Ie Tyr-96 → Phe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>73.6 ± 12.2</td>
<td>1.3 ± 0.1</td>
<td>$1.8 \times 10^4$</td>
<td>0.92</td>
<td>1.7</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>79.0 ± 11.1</td>
<td>1.1 ± 0.1</td>
<td>$1.3 \times 10^4$</td>
<td>1.6</td>
<td>4.2</td>
</tr>
<tr>
<td>Neamine</td>
<td>326 ± 35</td>
<td>1.3 ± 0.1</td>
<td>$3.9 \times 10^3$</td>
<td>0.34</td>
<td>1.4</td>
</tr>
<tr>
<td>Fortimicin A$^{b}$</td>
<td>43.5 ± 12.1</td>
<td>0.45 ± 0.04</td>
<td>$1.0 \times 10^4$</td>
<td>0.62</td>
<td>1.1</td>
</tr>
<tr>
<td>Paromomycin</td>
<td>771 ± 310</td>
<td>0.026 ± 0.006</td>
<td>$3.3 \times 10^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAC(6\textsuperscript{r})-Ie Asp-99 → Ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>45.3 ± 9.0</td>
<td>0.035 ± 0.002</td>
<td>$7.9 \times 10^2$</td>
<td>34</td>
<td>39</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>128 ± 28</td>
<td>0.033 ± 0.003</td>
<td>$2.6 \times 10^2$</td>
<td>52</td>
<td>215</td>
</tr>
<tr>
<td>Neamine</td>
<td>443 ± 89</td>
<td>0.029 ± 0.001</td>
<td>$6.5 \times 10^4$</td>
<td>45</td>
<td>15</td>
</tr>
<tr>
<td>Fortimicin A$^{b}$</td>
<td>ND</td>
<td>$2 \times 10^{-5}$</td>
<td>ND</td>
<td>1400</td>
<td>ND</td>
</tr>
<tr>
<td>Paromomycin$^{b}$</td>
<td>ND</td>
<td>$&lt;2 \times 10^{-5}$</td>
<td>ND</td>
<td>&gt;2500</td>
<td>ND</td>
</tr>
<tr>
<td>AAC(6\textsuperscript{r})-Ie Asp-99 → Asn</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>39.9 ± 9.0</td>
<td>0.17 ± 0.01</td>
<td>$4.4 \times 10^3$</td>
<td>6.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>80.2 ± 33</td>
<td>0.038 ± 0.005</td>
<td>$4.3 \times 10^3$</td>
<td>45</td>
<td>131</td>
</tr>
<tr>
<td>Neamine</td>
<td>509 ± 114</td>
<td>0.028 ± 0.003</td>
<td>$5.5 \times 10^3$</td>
<td>16</td>
<td>101</td>
</tr>
<tr>
<td>Fortimicin A$^{b}$</td>
<td>ND</td>
<td>$8 \times 10^{-5}$</td>
<td>ND</td>
<td>350</td>
<td>ND</td>
</tr>
<tr>
<td>Paromomycin$^{b}$</td>
<td>ND</td>
<td>$&lt;1 \times 10^{-5}$</td>
<td>ND</td>
<td>&gt;2500</td>
<td>ND</td>
</tr>
<tr>
<td>AAC(6\textsuperscript{r})-Ie Asp-99 → Glu</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>17.0 ± 6.3</td>
<td>0.11 ± 0.01</td>
<td>$6.5 \times 10^3$</td>
<td>11</td>
<td>4.8</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>102 ± 23</td>
<td>0.19 ± 0.02</td>
<td>$1.8 \times 10^4$</td>
<td>91</td>
<td>30</td>
</tr>
<tr>
<td>Neamine</td>
<td>229 ± 30</td>
<td>0.066 ± 0.005</td>
<td>$2.9 \times 10^4$</td>
<td>6.6</td>
<td>19</td>
</tr>
<tr>
<td>Fortimicin A$^{b}$</td>
<td>70.0 ± 28.1</td>
<td>0.006 ± 0.001</td>
<td>$8.2 \times 10^3$</td>
<td>49</td>
<td>134</td>
</tr>
<tr>
<td>Paromomycin$^{b}$</td>
<td>ND</td>
<td>$4 \times 10^{-5}$</td>
<td>ND</td>
<td>140</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^{a}$ Values taken from Ref. 13.

$^{b}$ Due to the low activities in the mutant proteins associated with these substrates, only an estimate of $k_{cat}$ could be determined where aminoglycoside was held at 1 mM and [\textsuperscript{14}C]acetyl-CoA was held at 200 mM.

$^{c}$ ND, not determined.

7.2–7.3) could be associated with a residue that needs to be deprotonated for maximum enzyme activity. The putative active site base required to deprotonate the antibiotic would normally need to be deprotonated to accept a hydrogen from the aminoglycoside substrate. Thus, the pH profiles are consistent with a chemical mechanism employing an active site base and an active site acid, and we can tentatively assign the first pK\textsubscript{a} of the active site base to the active site base.

Mutational Analyses of AAC(6\textsuperscript{r})-Ie Tyr-96 and Asp-99—These results suggest that AAC(6\textsuperscript{r})-Ie employs an active site base that acts to deprotonate the aminoglycoside substrate, thereby generating a more potent nucleophile to facilitate acetyl transfer from acetyl-CoA. In members of the GNAT superfamily, there are two amino acid positions that have been suggested to act as the active site base. In the histone acetyltransferase tGCN5, Glu-173 has been suggested to fulfill this role (30, 31), whereas in AANAT, the active site base appears to be His-122 (29). The three-dimensional structure of AAC(6\textsuperscript{r})-APH(2)\textsuperscript{a} has not been determined, so we used available sequence alignments (16, 40) to generate a partial alignment of GNAT family members that could aid in identifying the putative active site base in AAC(6\textsuperscript{r})-Ie (Fig. 4).

The residues in AAC(6\textsuperscript{r})-Ie that align with known or predicted active site bases in GNAT family members are Tyr-96 and Asp-99 (Fig. 4). To test the possibility that these residues could act as the active site base, we generated the following mutant proteins: AAC(6\textsuperscript{r})-Ie Tyr-96 → Phe, AAC(6\textsuperscript{r})-Ie Asp-99 → Ala, AAC(6\textsuperscript{r})-Ie Asp-99 → Asn, and AAC(6\textsuperscript{r})-Ie Asp-99 → Glu. The mutation of Asp-99 to Ala is a more drastic change, whereas mutation to Asn conserves R group length but not the proton extracting carboxylate moiety and mutation to Glu conserves the carboxylate moiety but extends R group length by one methylene unit.

There were only minor effects on the steady state kinetic parameters when Tyr-96 was mutated to Phe (Table I). The largest effects were seen for paromomycin (e.g. change in $k_{cat}/K_m$ compared with WT was 5.5-fold). These results are not consistent with Tyr-96 acting as an active site base, although it may have a minor effect on substrate binding with some aminoglycosides.

The changes in steady-state parameters for AAC(6\textsuperscript{r})-Ie Asp-99 → Ala and AAC(6\textsuperscript{r})-Ie Asp-99 → Asn were similar.
For kanamycin A, there were significant decreases in both $k_{\text{cat}}$ (45- to 52-fold) and $k_{\text{cat}}/K_m$ (131- to 215-fold) parameters. The changes for neamine were smaller but still significant for both $k_{\text{cat}}$ (15- to 16-fold) and $k_{\text{cat}}/K_m$ (85- to 101-fold). In contrast, there were more drastic changes for $k_{\text{cat}}$ with fortimicin A (350- to 1400-fold) and paromomycin ($>2500$).

The decreases in activity determined with AAC(6')-Ie Asp-99 $\rightarrow$ Glu were generally less than for either Ala or Asn mutations (Table I). This result suggests that the carboxylate moiety of Asp-99 is most critical for WT enzyme activity. This would be expected if Asp-99 acts as the active site base, because it would require the ability to ionize and accept a hydrogen from the aminoglycoside substrate. A Glu at this position would have a similar capacity, although it would not likely be optimally positioned for such a task, whereas Asn or Ala could not fulfill this role. Moreover, the fact that AAC(6')-Ie Asp-99 $\rightarrow$ Ala and AAC(6')-Ie Asp-99 $\rightarrow$ Asn become increasingly impaired in their ability to catalyze acetyl transfer with paromomycin is consistent with Asp-99 acting as the active site base, because it will become exceedingly important to have an active site base to deprotonate the aminoglycoside when the $pK_a$ of the acceptor group increases and a greater proportion of the substrate will be in a noncompetent form for efficient acetyl group transfer at physiological pH.

To correlate in vitro effects to biological impact, we performed minimum inhibitory concentration (MIC) determinations for *E. coli* expressing AAC(6')-APH(2') WT or Asp-99 mutant enzymes (Table IV). Because APH(2')-Ia cannot phosphorylate fortimicin A, protection of bacteria from this particular antibiotic is solely the responsibility of the AAC(6')-Ie activity in the bifunctional enzyme. The MIC determinations were consistent with the kinetic data, where *E. coli* carrying pET15AACAPH Asp-99 $\rightarrow$ Ala and pET15AACAPH Asp-99 $\rightarrow$ Asn provided no protection against fortimicin A (Table IV). This finding further underscores the importance of Asp-99 in the acetylation and detoxification of fortimicin A.

**pH Effects for AAC(6')-Ie Asp-99 $\rightarrow$ Ala**—We have previously assigned the first ionization in the pH profile of AAC(6')-Ie to the putative active site base. To further test this hypothesis and more fully develop the role of Asp-99 in the catalytic mechanism of AAC(6')-Ie, we determined the effects of pH on $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ for AAC(6')-Ie Asp-99 $\rightarrow$ Ala (Fig. 3C and Table III). The results do not show the double ionization curves as seen in AAC(6')-Ie WT. The pH curves for $k_{\text{cat}}$ with either neamine or acetyl-CoA as the variable substrate and for $k_{\text{cat}}/K_m$ with acetyl-CoA as the variable substrate show only a single ionization associated with the second $pK_a$ in AAC(6')-Ie WT and AAC(6')-Ie Asp-99 $\rightarrow$ Ala activity with the variable substrate acetyl-CoA (Table III). Thus, these results are consistent with Asp-99 serving as the active site base, and we can assign the first $pK_a$ in the pH profile of AAC(6')-Ie WT to Asp-99. When neamine is the variable substrate, there is not a distinct ionization (Table III). This is likely related to the form of
neamine that preferentially binds to AAC(6′)-Ie WT compared with AAC(6′)-Ie Asp-99 → Ala. With AAC(6′)-Ie WT, neamine is bound as the fully protonated form, however, as AAC(6′)-Ie Asp-99 → Ala lacks an active site base to deprotonate the incoming aminoglycoside, neamine likely binds in the unprotonated form in order to be chemically active. This effect complicates analysis and essentially conceals the basic limb of the pH profile.

Inhibition of AAC(6′)-Ie by (Halomethyl)naphthalene and (Halomethyl)phenanthrene Derivatives—A screen of a library of small planar molecules against AAC(6′)-Ie identified 1-(bromomethyl)phenanthrene and 9-(chloromethyl)phenanthrene as inhibitors of AAC(6′)-Ie, where 1-(bromomethyl)phenanthrene gave much more significant inhibition than 9-chloromethylphenanthrene (Fig. 5). This difference may be related to either the position or nature of the halogen group. We also assayed 1-(bromomethyl)naphthalene and 1-(chloromethyl)naphthalene to assess the importance of the halo-group and the additional benzene ring to inhibition by 1-(bromomethyl)phenanthrene. The bromomethyl-derivative demonstrated more significant inhibition of AAC(6′)-Ie activity than did the chloromethyl-derivative, however, 1-(bromomethyl)naphthalene was a much poorer inhibitor than was 1-(bromomethyl)phenanthrene (Fig. 5), highlighting the importance of both the bromo-group and the additional benzene ring in inhibition.
A linear plot of the first order rate constants versus enzyme activity. Site residue with a first order rate constant $k$ for 1-threne (Fig. 6A) and 1-(bromomethyl)naphthalene (not shown), Bthrene. Enzyme (0.2–2 M) was incubated with 40 M 1-(bromomethyl)phenanthrene for 10 min at 37 °C before the addition of acetyl-CoA (500 M) to assay for acetyltransferase activity. These inactivation reactions were conducted at different pH levels using the following buffers: 25 mM MOPS-NaOH (pH 6.5), 25 mM HEPES-NaOH (pH 7.0–8.0), and 25 mM glycylglycine (pH 8.5).

Neither extensive dialysis nor gel filtration could return enzyme activity following inactivation by 1-(bromomethyl)phenanthrene, again consistent with 1-(bromomethyl)phenanthrene acting as a covalent modifier of AAC(6’)-Ie. Moreover, matrix-assisted laser desorption ionization time-of-flight mass spectral analysis was consistent with covalent modification of the enzyme by 1-(bromomethyl)phenanthrene (data not shown). These results suggest that 1-(bromomethyl)phenanthrene inactivates AAC(6’)-Ie by covalently modifying an important residue in the enzyme.

1-(Bromomethyl)phenanthrene Inactivates AAC(6’)-Ie by Covalently Modifying Asp-99—To gain insight into which residue or residues are modified by 1-(bromomethyl)phenanthrene, we performed substrate/inhibitor protection experiments, where the enzyme was incubated with 1-(bromomethyl)phenanthrene and kanamycin A and/or desulfo-CoA prior to measurement of acetyl transfer activity with the addition of acetyl-CoA. Kanamycin A and desulfo-CoA both weakly protected AAC(6’)-Ie from inactivation by 1-(bromomethyl)phenanthrene (Fig. 7A). Moreover, when the enzyme was incubated with both kanamycin A and desulfo-CoA, protection was much more substantial and was additive with respect to the single compound protection experiments (Fig. 7A). This result is consistent with 1-(bromomethyl)phenanthrene having a binding site that overlaps that of both the kanamycin A and desulfo-CoA/acetyl-CoA binding sites.

Taking into account the results of the protection experiments, only a few select residues in AAC(6’)-Ie could be potential candidates to serve as the active site nucleophile in the reaction. Because our attempts to identify the residue through a series of experiments involving large-scale enzyme inactivation, proteolysis of the complex, and mass spectral analysis of the proteolytic fragments failed due to relative insolubility of 1-(bromomethyl)phenanthrene and nonspecific protein losses during work-up, we opted to use an alternative approach. We previously determined that, although 1-(bromomethyl)phenanthrene can inactivate AAC(6’)-Ie, it cannot inactivate AAC(6’)-
Its inability to inactivate AAC(6\(^{-}\))-Ii could be because 1-(bromomethyl)phenanthrene can not efficiently bind to AAC(6\(^{-}\))-Ii or because AAC(6\(^{-}\))-Ii lacks an appropriate active site residue to serve as the nucleophile in the reaction.

Close inspection of the active site of AAC(6\(^{-}\))-Ii, together with the alignment between AAC(6\(^{-}\))-Ii and AAC(6\(^{-}\))-Ie (Fig. 4), suggested that Asp-99 in AAC(6\(^{-}\))-Ie could be responsible for the different reactivities of the enzymes. The equivalent residue in AAC(6\(^{-}\))-Ii (His-74) would not act as a nucleophile in the reaction with 1-(bromomethyl)phenanthrene, and there does not appear to be any other candidate residues in the vicinity. To test this hypothesis, we determined the sensitivities of Tyr-96 and Asp-99 mutant proteins to 1-(bromomethyl)phenanthrene. AAC(6\(^{-}\))-Ie Tyr-96 \(\rightarrow\) Phe was just as sensitive as wild type enzyme to inactivation by 1-(bromomethyl)phenanthrene, indicating that the hydroxyl group of Tyr-96 is not required for inactivation (Fig. 7B). However, AAC(6\(^{-}\))-Ie Asp-99 \(\rightarrow\) Ala and AAC(6\(^{-}\))-Ie Asp-99 \(\rightarrow\) Asn were completely resistant to inactivation by 1-(bromomethyl)phenanthrene (Fig. 7B). This suggested that either Asp-99 is the active site residue that is modified by 1-(bromomethyl)phenanthrene or Asp-99 is important in the initial binding event between compound and enzyme. Consistent with the former hypothesis, AAC(6\(^{-}\))-Ie Asp-99 \(\rightarrow\) Glu is sensitive to inactivation, albeit less sensitive than AAC(6\(^{-}\))-Ie WT (Fig. 7B). This result suggests that the carboxylate moiety provided by Asp-99 (or Asp-99 \(\rightarrow\) Gln, Arg, Gln) is critical to the inactivation reaction and implicates Asp-99 as the active site residue that is covalently modified by 1-(bromomethyl)phenanthrene.

To further validate this conclusion, we determined the effect that pH has on the inactivation of AAC(6\(^{-}\))-Ie by 1-(bromomethyl)phenanthrene. AAC(6\(^{-}\))-Ie was most sensitive to inactivation at lower pH, whereas it became more resistant as the assay pH increased (Fig. 8). This result yielded a pK\(_{a}\) of 7.2. We have previously assigned a pK\(_{a}\) of 7.2 to Asp-99, thus, together these results are consistent with Asp-99 serving as the active site nucleophile that is covalently modified by 1-(bromomethyl)phenanthrene. Therefore, although the presence of Asp-99 in AAC(6\(^{-}\))-Ie leads to a wider resistance profile by enabling AAC(6\(^{-}\))-APH(2\(^{-}\)) to acetylate fortimicin A and paromomycin, it also makes the enzyme sensitive to inactivation by 1-(bromomethyl)phenanthrene.

Molecular Mechanism of AAC(6\(^{-}\))-Ie and Comparison to Other GNAT Family Members—Because there is no available crystal structure for AAC(6\(^{-}\))-APH(2\(^{-}\)), it is not known if Asp-99 acts directly upon aminoglycoside substrate or if base catalysis is mediated through a chain of water molecules similar to that proposed for both tGCN5 histone acetyltransferase and AANAT (17). However, recently the structure of AAC(2\(^{-}\))-Ic from Mycobacterium tuberculosis, an aminoglycoside acetyltransferase also known to catalyze O-acetylation, has been solved (15). As with other GNAT superfamily members, there is not a direct interaction with the substrate 2'-NH$_2$ and a potential active site base, but rather, a series of water molecules that connects the amine to Ghu-82, the residue that aligns with Asp-99 in AAC(6\(^{-}\))-Ie. This suggests that with a sufficiently potent active site base, O-acetylation may be catalyzed through a “proton-wire” of appropriately positioned water molecules.

These studies identify Asp-99 as an active site base required for O-acetylation of aminoglycoside substrates and N-acetylation of fortimicin A and provides the molecular basis for these unique properties in an AAC(6\(^{-}\)). It also identifies this residue as sensitive to modification by small molecule inhibitors. This provides a unique anchoring site for the development of specific inhibitors of AAC(6\(^{-}\))-Ie that could find use as leads in the development of anti-resistance molecules.
The Molecular Basis of the Expansive Substrate Specificity of the Antibiotic Resistance Enzyme Aminoglycoside Acetyltransferase-6′-Aminoglycoside Phosphotransferase-2": THE ROLE OF ASP-99 AS AN ACTIVE SITE BASE IMPORTANT FOR ACETYL TRANSFER

David D. Boehr, Stephen I. Jenkins and Gerard D. Wright

doi: 10.1074/jbc.M211680200 originally published online February 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211680200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 13 of which can be accessed free at http://www.jbc.org/content/278/15/12873.full.html#ref-list-1