A Random Sequential Mechanism of Aminoglycoside Acetylation by Mycobacterium tuberculosis Eis Protein

Oleg V. Tsodikov*, Keith D. Green, Sylvie Garneau-Tsodikova*

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, BioPharm Complex, Lexington, Kentucky, United States of America

Abstract

An important cause of bacterial resistance to aminoglycoside antibiotics is the enzymatic acetylation of their amino groups by acetyltransferases, which abolishes their binding to and inhibition of the bacterial ribosome. Enhanced intracellular survival (Eis) protein from Mycobacterium tuberculosis (Mt) is one of such acetyltransferases, whose upregulation was recently established as a cause of resistance to aminoglycosides in clinical cases of drug-resistant tuberculosis. The mechanism of aminoglycoside acetylation by MtEis is not completely understood. A systematic analysis of steady-state kinetics of acetylation of kanamycin A and neomycin B by Eis as a function of concentrations of these aminoglycosides and the acetyl donor, acetyl coenzyme A, reveals that MtEis employs a random-sequential bisubstrate mechanism of acetylation and yields the values of the kinetic parameters of this mechanism. The implications of these mechanistic properties for the design of inhibitors of Eis and other aminoglycoside acetyltransferases are discussed.

Introduction

The emergence and spread of multidrug-resistant bacteria is a worldwide problem that requires deep understanding of the resistance mechanisms to develop novel rational approaches to antibacterial therapy. There are several mechanisms of bacterial drug resistance and one is chemical drug modification by the pathogen. Mycobacterium tuberculosis (Mt) is a notoriously pervasive infectious bacterium, whose multidrug-resistant strains are steadily spreading globally. A large fraction of clinical isolates of Mt tuberculosis that are resistant to a second-line drug used in treatment of extensively drug-resistant tuberculosis. The acetylating activity of kanamycin A (KAN), do not bear any ribosomal mutations that weaken inhibition by AGs [1,2]. Instead, these strains harbor upregulating mutations in the promoter of the eis (enhanced intracellular survival) gene encoding an AG acetyltransferase, Eis [1]. KAN acetylated by MtEis does not have any antibacterial activity [1,3]. MtEis, unlike other AG acetyltransferases, is capable of acetylating AGs efficiently at several amino groups, thus displaying a unique regioselectivity and strong AG inactivation properties [4]. Several AGs that are used in clinic and the second-line drug neomycin are rendered inactive by the acetylating activity of MtEis [5]. Moreover, some acetylation positions are unique to this enzyme, since they are not modified by other acetyltransferases [3]. Because Eis homologs are found in many other bacteria in addition to mycobacteria, they pose a formidable challenge as an AG resistance factor. Moreover, because of the broad substrate versatility of Eis, a traditional approach to overcoming Eis-based resistance by designing a novel AG antibiotic that cannot be acetylated by Eis, is not likely to succeed.

The kinetic mechanism of AG acetylation by MtEis remains incompletely understood. Similarly to other acetyltransferases, MtEis uses acetyl coenzyme A (AcCoA) as the acetyl group donor and, therefore, MtEis is a bisubstrate enzyme. The AG substrate and the AcCoA can bind the enzyme either in a strict order or randomly to form the preacetylation ternary complex. While some AG acetyltransferases were demonstrated to obey a random sequential mechanism [8–11], others employ an ordered sequential mechanism where AcCoA needs to bind the enzyme first followed by the AG [12–14], and some of the enzymes follow one or the other mechanism depending on the AG scaffold [15]. A mechanism where the AG must bind the enzyme first has not been reported yet, to our knowledge. We recently demonstrated that a homolog of MtEis from Mycobacterium smegmatis obeys the random sequential mechanism of KAN acetylation [6]. Here, we report a systematic kinetic analysis of the clinically relevant enzyme, MtEis, with two AGs, KAN and neomycin B (NEO). KAN, a member of the 4,6-disubstituted 2-deoxystreptamine family of AGs, was selected because it is a second-line drug used in treatment of extensively drug-resistant tuberculosis. NEO was chosen as a representative of another major family of AGs, the 4,5-disubstituted 2-deoxystreptamine, in order to test if the mechanism of acetylation by MtEis is dependent on the molecular scaffold of the drug.

Materials and Methods

Materials

MtEis was expressed and purified as previously reported [4]. KAN, NEO, and AcCoA were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without any further purification.
Steady-state acetyltransferase assays

Reactions were carried out in Tris buffer (50 mM, pH 8.0) using varying concentrations of KAN or NEO (0, 20, 50, 100, 250, 500, 1000, and 2000 μM) at several concentrations of AcCoA (25, 50, 100, 200, 300, and 500 μM) using constant concentrations of Eis (0.25 μM) and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, 2 mM). Reactions were monitored using a SpectraMax M5 multimode plate reader by taking absorbance measurements at 412 nm every 15 s for 15 min. Initial rates were calculated using the first 1.5 min of the reaction.

Analysis of the bisubstrate kinetics of AG acetylation by MtEis

A random sequential rapid equilibrium bisubstrate mechanism is given by the following kinetic scheme [6]:

\[
\begin{align*}
E + AG + AcCoA & \Rightarrow E \cdot AG + AcCoA \Rightarrow E \cdot AG \cdot AcCoA \\
E + AG + AcCoA & \Rightarrow E \cdot AG \cdot AcCoA + AG \\
E \cdot AG \cdot AcCoA & \Rightarrow AG - Ac + CoA + E,
\end{align*}
\]

where E designates the enzyme, AG and AG-Ac is the aminoglycoside and its acetylated form, respectively, and AcCoA and CoA are acetyl coenzyme A and coenzyme A, respectively. Then, under pseudo-first order conditions (large excess of the two substrates over the enzyme), the apparent Michaelis-Menten parameters in terms of the microscopic mechanism parameters are:

\[
k_{cat, AG} = \frac{k_{cat}[AcCoA]}{K_d,AG + [AcCoA]} \tag{2}
\]

\[
K_{m,AG} = K_{d,AG} \frac{K_{d,AG}(E \cdot AG)}{[AcCoA]} \frac{K_{d,AG}(E \cdot AG + [AcCoA])}{[AcCoA]} \tag{3}
\]

\[
k_{cat, AcCoA} = \frac{k_{cat}[AG]}{K_d, AcCoA(E \cdot AG) + [AG]} \tag{4}
\]

\[
K_{m,AcCoA} = K_{d,AcCoA} \frac{K_{d,AcCoA}(E \cdot AcCoA)}{[AG]} \frac{K_{d,AcCoA}(E \cdot AcCoA) + [AG]}{[AG]} \tag{5}
\]

Here, the subscript “AG” for \(k_{cat}\) and \(K_m\) means that this constant is obtained from the dependence of the steady-state rate on the concentration of AG measured at a fixed concentration of AcCoA. The analogous nomenclature is used for AcCoA. For the subscript nomenclature of equilibrium binding constants \(K_d\), for example, \(K_{d,AG}(E \cdot AcCoA)\) is the equilibrium constant for binding of AG to E·AcCoA complex, etc; \(k_{cat}\) is the microscopic rate constant of the acetylation step (last equation in scheme (1)).

In the scheme for the ordered mechanism in which AcCoA must bind the enzyme first, the above parameters have the following functional form:

\[
k_{cat, AG} = k_{cat} \tag{6}
\]

\[
K_{m,AG} = K_{d,AG} \left(1 + \frac{K_{d,AcCoA}}{[AcCoA]}\right) \tag{7}
\]

The ordered mechanism is ruled out by the observed kinetics, as described in the Results and Discussion section. Because the kinetics were measured in sets of experiments at different fixed concentrations of AcCoA for each concentration of AcCoA varying the concentration of AG, a statistically rigorous way of determining the microscopic mechanism constants \(k_{cat}\) and \(K_m\) from the data is to determine the apparent Michaelis-Menten constants \(k_{cat, AG}\) and \(K_{m, AG}\) first. The next step is to fit dependence of \(k_{cat, AG}\) on [AcCoA] described by eq. (2) to the respective observed values, to obtain \(k_{cat}\) and \(K_{d, AG} + K_{d, AcCoA}\). These values are obtained for each AG independently. Finally, because \(K_{d, AcCoA}(E)\) is AG-independent, its value is determined by a nonlinear regression data fitting of eq. (3) to the observed \(K_{m, AG}\) dependence on [AcCoA] for KAN and NEO together. In the same fitting procedure two independent values \(K_{d, AG} + K_{d, AcCoA}\) for KAN and NEO are obtained. This analysis was performed by nonlinear regression with SigmaPlot 11.0 (Systat). We have observed a preparation-dependent activity of Eis, varying within a 4-fold range. For this reason, all experiments in this study were performed with the same preparation of MtEis. The differences in fraction of active MtEis translate in corresponding differences in values of \(k_{cat}\) reported in this and other studies. Values of \(K_m\) and \(K_d\) as well as the relative differences in \(k_{cat}\) values for different AGs are not affected by this variability.

Results and Discussion

Steady-state kinetic measurements of KAN and NEO acetylation by MtEis

In order to distinguish among the two ordered sequential mechanisms and a random sequential mechanism of binding of the AG and the AcCoA to MtEis to form a ternary acetylation complex, we performed a series of steady-state acetylation kinetic assays as a function of two independent variables, the concentrations of AG and AcCoA, both in large excess of the enzyme. These experiments were carried out with two AGs, KAN and NEO. For both KAN and NEO, the steady-state rate of acetylation by MtEis followed a hyperbolic dependence when plotted as a function of concentration of AG at a fixed concentration of AcCoA (Figures 1A and 2A for KAN and NEO, respectively) or as a function of concentration of AcCoA at a fixed concentration of AG (Figures 1B and 2B for KAN and NEO, respectively). Each of such hyperbolic dependences for KAN and NEO, where the concentration of AG is an independent variable (Figures 1A and 2A, respectively) yields apparent Michaelis-Menten constants, \(K_{m, AG}\) and \(k_{cat, AG}\). Dependence of these constants on the concentration of AcCoA is also hyperbolic within the experimental uncertainty (Figures 1C, 1D for KAN and Figures 2C, 2D for NEO). We observe that \(K_{m, AG}\) increases with increasing concentration of AcCoA (Figures 1D and 2D) for both KAN and NEO; this is possible only for a random sequential mechanism (scheme (1), eq. (3)), where \(k_{d, AG}(E \cdot AG) < k_{d, AG}(E \cdot AcCoA)\), i.e., AcCoA has a higher affinity towards free enzyme than towards AG bound enzyme. In other words, binding of AcCoA and AG is anti-cooperative. A similar observation was made recently about KAN...
Acetylation of NEO as described in the text.

The rapid equilibrium mechanism of acetylation by MtEis makes physical sense, when one considers the ability of MtEis to efficiently acetylate AGs at multiple amino groups. This random sequential mechanism would allow an AG bound to MtEis to simply change its orientation in the active site after one acetylation, independently of dissociation of the CoA product and binding of another AcCoA for subsequent acetylation of the same AG. In contrast, if, for example, AG binding strictly followed AcCoA binding, the AG would need to dissociate after each acetylation event in order to rebind the same or another enzyme bound to AcCoA.

The quantitative analysis of these kinetic data in terms of the random sequential mechanism yields microscopic Michaelis–Menten parameter values for KAN and NEO. We obtain similar values of $k_{cat}$ for KAN and NEO ($k_{cat} = 0.68 \pm 0.15 \text{ s}^{-1}$ and $0.86 \pm 0.16 \text{ s}^{-1}$) (Figure 1A, 1C and Figure 2A, 2C) and a somewhat higher affinity of AcCoA to the KAN bound enzyme than to the NEO bound enzyme ($K_{d,AG}(E) = 18 \pm 14 \text{ M}$ and $111 \pm 62 \text{ M}$, for KAN and NEO, respectively). On the other hand, NEO binds the AcCoA bound enzyme with an approximately 3-fold higher affinity than KAN (Figures 1D and 2D); the values of the equilibrium binding constants obtained from the simultaneous fit of the KAN and NEO acetylation data (see Materials and Methods) are $K_{d,AG}(E) = 0.16 \pm 0.03 \text{ s}^{-1}$ and $111 \pm 62 \text{ M}$ for KAN and NEO, respectively. This analysis also yields the equilibrium constant for binding of AcCoA to MtEis, $K_{d,AG}(E) = 18 \pm 14 \text{ M}$. It is to note that this value of $K_{d,AG}(E)$ is about 4-fold lower than that for AcCoA binding to Eis from M. smegmatis reported recently [6] and not equal to it, as it was assumed. Because the four equilibria in scheme (1) form a thermodynamic cycle, any three $K_{d}$ values yield the fourth one, in this case the equilibrium constant for binding of AG to free enzyme, $K_{d,AG}(E) = K_{d,ACOA(AG)}E/E_{is} = 176 \pm 44 \text{ M}$ and $22 \pm 17 \text{ M}$ for KAN and NEO, respectively. These results demonstrate quantitatively the anti-cooperativity of AcCoA and AG binding, as discussed above based on qualitative grounds. A different, four-ring structure of NEO from the three-ring structure of KAN may explain stronger binding of NEO to MtEis. In addition, NEO is tri-acetylated by MtEis while KAN is di-acetylated [3,4], indicating that NEO binds MtEis in more orientations than KAN does, which may explain the higher affinity of NEO to MtEis.

**Relationship to other AG acetyltransferases and considerations for the design of MtEis inhibitors**

The random sequential mechanism is more common among characterized AG acetyltransferases than the ordered sequential mechanism where AcCoA binds the enzyme first. The ability of either AG or AcCoA to bind the free enzyme raises a possibility that bisubstrate compounds combining the chemical features of both substrates can be developed as MtEis inhibitors, which would be more potent than respective substrate analog inhibitors individually or as a combination. The 4,5-disubstituted 2-deoxystreptamine four-ring scaffold of NEO appears to be more promising than the 4,6-disubstituted 2-deoxystreptamine three-ring scaffold of KAN for such design, based on the stronger affinity of NEO to MtEis. Bisubstrate inhibitors of GCN5-related $\alpha$-acetyltransferases have been reported [16–21]. Blanchard and colleagues developed and elegantly applied analysis of the inhibition kinetics by such inhibitors to the dissection of the kinetic mechanism, an alternative to the analysis presented in this.
acetylation of NEO on the concentration of AcCoA at different concentrations of NEO, as specified. C. Dependence of the apparent rate constant ($k_{cat,AG}$), as obtained from data shown in panel A, on the concentration of AcCoA. D. Dependence of the apparent $K_m$, as obtained from data shown in panel A, on the concentration of AcCoA. The theoretical curve in D is the best simultaneous fit of eq. (3) to these values and those for acetylation of KAN as described in the text.

Figure 3. Steady-state kinetics of NEO acetylation by MtEis and their analysis. A. Representative dependences of the steady-state rate of acetylation of NEO on the concentration of NEO at different concentrations of AcCoA, as specified. B. Representative dependences of the steady-state rate of acetylation of NEO on the concentration of AcCoA at different concentrations of NEO, as specified. C. Dependence of the apparent rate constant ($k_{cat,AG}$), as obtained from data shown in panel A, on the concentration of AcCoA. D. Dependence of the apparent $K_m$, as obtained from data shown in panel A, on the concentration of AcCoA. The theoretical curve in D is the best simultaneous fit of eq. (3) to these values and those for acetylation of KAN as described in the text.

doI:10.1371/journal.pone.0092370.g002

study [22]. Consistent with the thermodynamic argument made above, a bisubstrate inhibitor of the E. coli AAC(3)-IV acetyltransferase, which obeys the random sequential mechanism, is extremely strong, and its intrinsic $K_i$ could be obtained only by extrapolation [22]. These examples demonstrate the power of bisubstrate inhibitors as chemical probes. Even though therapeutically useful bisubstrate inhibitors of acetyltransferase targets have not emerged yet, examples of bisubstrate inhibitors of other enzymes that are used in clinic exist [22]. Development of a potent bisubstrate inhibitor of MtEis as a selective probe or a pharmaceutical lead, based on its unique structure and catalytic properties, is an attractive direction for future studies. Studies focusing on the development of such bisubstrate inhibitors of MtEis are currently underway in our laboratories.

Author Contributions
Conceived and designed the experiments: OVT KDG SGT. Performed the experiments: OVT KDG. Analyzed the data: OVT KDG SGT. Contributed reagents/materials/analysis tools: OVT KDG SGT. Wrote the paper: OVT SGT.

References
1. Zaunbrecher MA, Sikes RD Jr, Metchock B, Shimnick TM, Posey JE (2009) Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A 106: 20004–20009.
2. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, et al. (2011) Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. Antimicrob Agents Chemother 55: 2032–2041.
3. Houghton JL, Biswas T, Chen W, Tsodikov OV, Garneau-Tsodikova S (2013) Chemical and structural insights into the regioversatility of the aminoglycoside acetyltransferase Eis. ChemBioChem 14: 2127–2135.
4. Chen W, Biswas T, Porter VR, Tsodikov OV, Garneau-Tsodikova S (2011) Unusual regioversatility of acetyltransferase Eis, a cause of drug resistance in XDR-TB. Proc Natl Acad Sci U S A 108: 9804–9808.
5. Houghton JL, Green KD, Pricer RE, Mayhoub AS, Garneau-Tsodikova S (2013) Unexpected N-acetylation of capreomycyin by mycobacterial Eis enzymes. J Antimicrob Chemother 68: 800–805.
6. Chen W, Green KD, Tsodikov OV, Garneau-Tsodikova S (2012) Aminoglycoside multiacetylating activity of the enhanced intracellular survival protein from Mycobacterium smegmatis and its inhibition. Biochemistry 51: 4959–4967.
7. Pricer RE, Houghton JL, Green KD, Mayhoub AS, Garneau-Tsodikova S (2012) Biochemical and structural analysis of aminoglycoside acetyltransferase Eis from Anaerobiospirillum succiniciproducens. Mol Biosyst 8: 3305–3313.
8. Martel A, Masson M, Moreau N, Le Goffic F (1983) Kinetic studies of aminoglycoside acetyltransferase and phosphotransferase from Staphylococcus aureus RPAL. Relationship between the two activities. Eur J Biochem 133: 515–521.
9. Magnet S, Lambert T, Courvalin P, Blanchard JS (2001) Kinetic and mutagenic characterization of the chromosomally encoded Salmonella enterica AAC(6’)-Ib aminoglycoside N-acetyltransferase. Biochemistry 40: 3700–3709.
10. Magalhaes ML, Blanchard JS (2005) The kinetic mechanism of AAC3-IV aminoglycoside acetyltransferase from Escherichia coli. Biochemistry 44: 16275–16283.
11. Hegde SS, Javid-Majd F, Blanchard JS (2001) Overexpression and mechanistic analysis of chromosomally encoded aminoglycoside 2’-O-acetyltransferase (AAC(2’)-Ie) from Mycobacterium tuberculosis. J Biol Chem 276: 45876–45881.
12. Kim C, Villegas-Estrada A, Hesek D, Mobashery S (2007) Mechanistic characterization of the bifunctional aminoglycoside-modifying enzyme AAC3(9)/Ib/AAC(6’)-Ib’ from Pseudomonas aeruginosa. Biochemistry 46: 5270–5282.
13. Draker KA, Northrop DB, Wright GD (2003) Kinetic mechanism of the GCN5-related chromosomal aminoglycoside acetyltransferase AAC38’-Ii from Enterococcus faecalis: evidence of dimer subunit cooperativity. Biochemistry 42: 6565–6574.
14. Kim C, Hesek D, Zajicek J, Vakulenko SR, Mobashery S (2006) Characterization of the bifunctional aminoglycoside-modifying enzyme ANT(3′5′)-Ii/AAC(6′)-IId from Serratia marcescens. Biochemistry 45: 8368–8377.
15. Williams JW, Northrop DB (1978) Kinetic mechanisms of gentamicin acetyltransferase I: Antibiotic-dependent shift from rapid to nonrapid equilibrium random mechanisms. J Biol Chem 253: 5902–5907.
16. Gao F, Yan X, Auclair K (2009) Synthesis of a phosphonate-linked aminoglycoside-enzyme a bisubstrate and use in mechanistic studies of an enzyme involved in aminoglycoside resistance. Chemistry 15: 2064–2070.
17. Gao F, Yan X, Zahr O, Larsen A, Vong K, et al. (2008) Synthesis and use of sulfonamide-, sulfoxide-, or sulfone-containing aminoglycoside-CoA bisubstrates as mechanistic probes for aminoglycoside N6'-acetyltransferase. Bioorg Med Chem Lett 18: 5518–5522.
18. Williams JW, Northrop DB (1979) Synthesis of a tight-binding, mult subs ute analog inhibitor of gentamicin acetyltransferase I. J Antibiot (Tokyo) 32: 1147–1154.
19. Khalil EM, Cole PA (1998) A potent inhibitor of the melatonin rhythm enzyme. J Am Chem Soc 120: 6195–6196.
20. Gao F, Yan X, Baettig OM, Berghuis AM, Auclair K (2005) Regio- and chemoselective 6-N-derivatization of aminoglycosides: bisubstrate inhibitors as probes to study aminoglycoside 6'-N-acetyltransferases. Angew Chem Int Ed Engl 44: 6859–6862.
21. Poux AN, Cebrat M, Kim CM, Cole PA, Marmorstein R (2002) Structure of the GCN5 histone acetyltransferase bound to a bisubstrate inhibitor. Proc Natl Acad Sci U S A 99: 14065–14070.
22. Yu M, Magalhaes ML, Cook PF, Blanchard JS (2006) Bisubstrate inhibition: Theory and application to N-acyt yltransferases. Biochemistry 45: 14788–14794.