

Cosubstrate Tolerance of the Aminoglycoside Resistance Enzyme Eis from *Mycobacterium tuberculosis*

Wenjing Chen,^{b,c} Keith D. Green,^c and Sylvie Garneau-Tsodikova^{a,b,c}

Department of Medicinal Chemistry,^a Chemical Biology Doctoral Program,^b and Life Sciences Institute,^c University of Michigan, Ann Arbor, Michigan, USA

We previously demonstrated that aminoglycoside acetyltransferases (AACs) display expanded cosubstrate promiscuity. The enhanced intracellular survival (Eis) protein of *Mycobacterium tuberculosis* is responsible for the resistance of this pathogen to kanamycin A in a large fraction of clinical isolates. Recently, we discovered that Eis is a unique AAC capable of acetylating multiple amine groups on a large pool of aminoglycoside (AG) antibiotics, an unprecedented property among AAC enzymes. Here, we report a detailed study of the acyl-coenzyme A (CoA) cosubstrate profile of Eis. We show that, in contrast to other AACs, Eis efficiently uses only 3 out of 15 tested acyl-CoA derivatives to modify a variety of AGs. We establish that for almost all acyl-CoAs, the number of sites acylated by Eis is smaller than the number of sites acetylated. We demonstrate that the order of *n*-propionylation of the AG neamine by Eis is the same as the order of its acetylation. We also show that the 6' position is the first to be *n*-propionylated on amikacin and netilmicin. By sequential acylation reactions, we show that AGs can be acetylated after the maximum possible *n*-propionylation of their scaffolds by Eis. The information reported herein will advance our understanding of the multiacetylation mechanism of inactivation of AGs by Eis, which is responsible for *M. tuberculosis* resistance to some AGs.

The tuberculosis (TB) epidemic, caused primarily by *Mycobacterium tuberculosis*, kills millions of people worldwide each year. Taking into account the 2 billion currently infected and the current rate of infection, nearly 10 million people will become infected in the next year (15). The overuse of some drugs and the failure to comply with a proper therapeutic regimen, through either complete lack or intermittent availability of drugs and ineffective secondary care, have in part been responsible for the emergence of multidrug-resistant (MDR) *M. tuberculosis* strains, resistant to the first-line antibiotics isoniazid and rifampin, as well as extensively drug-resistant (XDR) *M. tuberculosis* strains that additionally render second-line anti-TB drugs ineffective, including the aminoglycosides (AGs) kanamycin A (KAN) and amikacin (AMK) (7, 17).

Resistance to the broad-spectrum AG antibiotics is a continuously increasing problem for the treatment of many serious bacterial infections (22). AG resistance results, in great part, from the evolution or acquisition of AG-modifying enzymes (AMEs) that acetylate (AG acetyltransferases [AACs]), phosphorylate (AG phosphotransferases [APHs]), or nucleotidylate (AG nucleotidyltransferases [ANTs]) various positions on the AG scaffolds, resulting in their deactivation as antibacterials (35). To broaden their AG resistance profile, bacteria have also evolved bifunctional AMEs, including AAC(6')-30/AAC(6')-Ib (46), AAC(6')-Ie/APH(2'')-Ia (2, 6), AAC(3)-Ib/AAC(6')-Ib' (14, 19, 25), and ANT(3'')-Ii/AAC(6')-IId (9, 24).

For a large fraction of *M. tuberculosis* clinical isolates, it was recently shown that upregulation of the enhanced intracellular survival (Eis) protein confers KAN resistance on the mycobacterium, a hallmark of XDR-TB (8, 45). This enzyme was initially found to be involved in intracellular survival of *Mycobacterium smegmatis* within the human macrophage-like cell line U-937 (42). Phase separation assays suggested that Eis appears primarily in the cytoplasm and in modest amounts in the cell envelope and in the culture supernatant (13). Further studies suggested that Eis inhibits T-cell proliferation *in vitro*, as well as subsequent production of tumor necrosis factor- α and interleukin-4 (28, 36). It was

suggested that Eis is a mycobacterial effector that is released into the host cell to modulate macrophage autophagy, inflammatory responses, and cell death via a reactive oxygen species (ROS)-dependent pathway (37). This year, Kim et al. found that Eis is capable of acetylating Lys55 of dual-specificity protein phosphatase 16 (DUSP16)/mitogen-activated protein kinase phosphatase-7 (MKP-7), a Jun N-terminal protein kinase (JNK)-specific phosphatase (26). They proposed that Eis of *M. tuberculosis* suppresses JNK-dependent autophagy, phagosome maturation, and ROS generation through inhibition of lipopolysaccharide (LPS)-induced JNK phosphorylation via acetylation of DUSP16/MKP-7. We recently discovered that Eis is a unique AAC that can modify multiple amine functionalities on a variety of AG scaffolds (10). This novel enzyme and its homolog in *M. smegmatis* (11) are, to date, the only known monofunctional AMEs capable of catalyzing multiacetylation reactions. For this study, it is important to distinguish acetylation, which exclusively refers to the transfer of an acetyl group, from acylation, which refers to the general transfer of any acyl moiety (e.g., *n*-propionyl, malonyl, crotonyl, etc., including acetyl).

We previously demonstrated that AACs could display broad acyl-coenzyme A (CoA) cosubstrate promiscuity (20, 21, 34). Here, to gain insight into the mechanism of multiacetylation of Eis, we performed an in-depth study of the cosubstrate tolerance of Eis. Through sequential use of acyl-CoAs, we established the limits of Eis cosubstrate tolerance. We also investigated the effect

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Address correspondence to Sylvie Garneau-Tsodikova, sylviegt@umich.edu.

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that the nature of different acyl-CoA derivatives has on the multiplicity of acylation.

MATERIALS AND METHODS

Materials and instrumentation. Eis and the AAC(6′)-Ie/APH(2′′)-Ia, AAC(3)-IV (30), and AAC(2′)-Ic (39) enzymes were expressed and purified as previously described (10, 20). AAC(6′)-Ie/APH(2′′)-Ia was used solely for its AAC(6′) activity and is referred to as AAC(6′) herein. The acyl-CoAs (acetyl-CoA [AcCoA], acetoacetyl-CoA, benzoyl-CoA, *n*-butyryl-CoA, crotonyl-CoA [CroCoA], glutaryl-CoA, *D,L*-β-hydroxybutyryl-CoA, isovaleryl-CoA, malonyl-CoA [MalCoA], palmitoyl-CoA, *n*-propionyl-CoA [ProCoA], hexanoyl-CoA, lauroyl-CoA, octanoyl-CoA, succinyl-CoA, and decanoyl-CoA) (see Fig. S1 in the supplemental material), 4,4′-dithiodipyridine (DTDP), ammonium molybdate, ammonium cerium nitrate, and the AGs (AMK, hygromycin [HYG], KAN, neomycin B [NEO], paromomycin [PAR], ribostamycin [RIB], sisomicin [SIS], and tobramycin [TOB]) (see Fig. S1 in the supplemental material) were purchased from Sigma-Aldrich (Milwaukee, WI). The AGs neamine (NEA) and netilmicin (NET) were purchased from AK Scientific (Mountain View, CA) (see Fig. S1). Ninety-six-well plates were purchased from Thermo Fisher Scientific (Waltham, MA). UV-Vis assays were monitored on a SpectraMax M5 plate reader. Liquid chromatography-mass spectrometry (LC-MS) was performed on a Shimadzu LCMS-2019EV equipped with a SPD-20AV UV-Vis detector and an LC-20AD liquid chromatograph.

Determination of cosubstrate profile for Eis by UV-Vis assays. To determine which CoA derivatives were cosubstrates for Eis, 15 CoA derivatives (acetoacetyl-CoA, benzoyl-CoA, *n*-butyryl-CoA, CroCoA, glutaryl-CoA, *D,L*-β-hydroxybutyryl-CoA, isovaleryl-CoA, MalCoA, palmitoyl-CoA, ProCoA, hexanoyl-CoA, lauroyl-CoA, octanoyl-CoA, succinyl-CoA, and decanoyl-CoA) were tested against several AGs (KAN, NEO, NET, SIS, and TOB). Reaction mixtures with AcCoA were used as positive controls. The acylation activity of Eis was monitored at 324 nm ($\epsilon_{324} = 19,800 \text{ M}^{-1} \text{ cm}^{-1}$) by a UV-Vis assay using DTDP, as previously reported (20). Reaction mixtures (200 μl) containing a CoA derivative (0.5 mM, 5 eq), AG (0.1 mM, 1 eq), DTDP (2 mM), and Tris-HCl (50 mM, pH 8.0, adjusted at room temperature [RT]), were initiated by the addition of Eis (0.5 μM) at 25°C. Reactions were monitored by taking readings every 30 s for 60 min in 96-well plates. In addition to AcCoA, only CroCoA, MalCoA, and ProCoA were found to be cosubstrates of Eis and were further studied with all AGs (AMK, HYG, KAN, NEA, NEO, NET, PAR, RIB, SIS, and TOB) as described below.

Determination of cosubstrate profile for Eis by LC-MS. By using LC-MS, the results obtained by UV-Vis assays were confirmed and the degree of acylation was determined for all 10 AG substrates. Reaction mixtures (30 μl) containing AG (0.67 mM, 1 eq), CoA derivatives (3.35 mM, 5 eq), Tris-HCl (50 mM, pH 8.0, adjusted at RT), and Eis (10 μM) were incubated at RT for 48 h. The Eis protein was then precipitated by the addition of ice-cold methanol (MeOH) (30 μl) to the reaction mixture, which was then kept at -20°C for at least 20 min. The precipitated protein was removed by centrifugation (13,000 rpm, RT, 10 min). The supernatant (10 μl) was diluted with H₂O (20 μl) and loaded onto the LC-MS. The masses of the acylated AGs present in each sample were determined in positive mode using H₂O (0.1% formic acid). Mass spectra of all AGs modified by Eis are shown in Fig. 1; also see Fig. S2, S3, and S4 and Table S2 in the supplemental material.

Steady-state kinetic measurements for CoA derivatives. The kinetic parameters for AcCoA and ProCoA were determined against 10 AGs (AMK, KAN, HYG, NEA, NEO, NET, PAR, RIB, SIS, and TOB) in reaction mixtures (100 μl) containing a fixed concentration of AG (0.5 mM), various concentrations of CoA derivatives (0, 20, 50, 100, 250, and 500 μM), and fixed concentrations of DTDP (2 mM), Tris-HCl (50 mM, pH 8.0, adjusted at RT), and Eis (0.25 μM). Using similar reaction conditions, the kinetic parameters for MalCoA and CroCoA were determined in reaction mixtures (100 μl) containing a fixed concentration of NEO (1

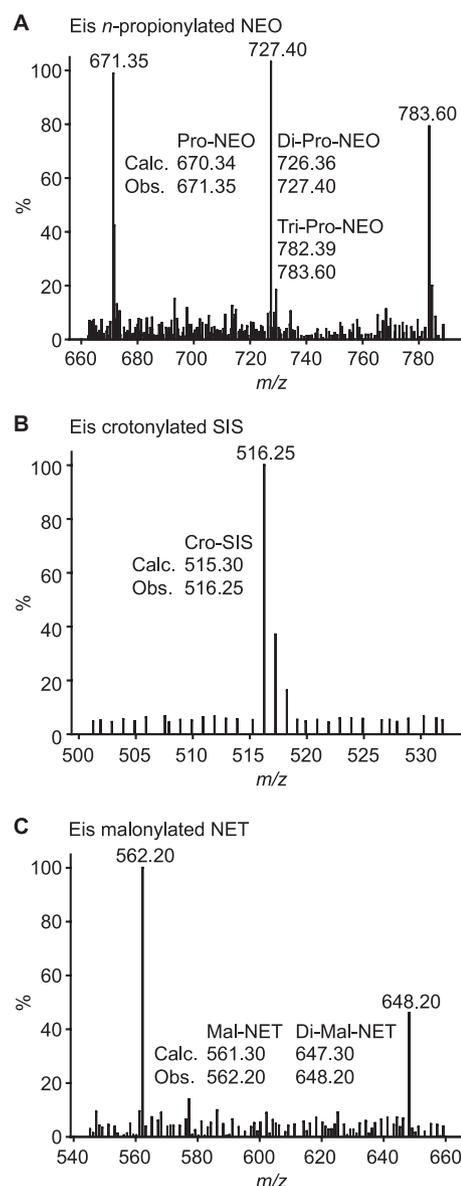


FIG 1 Representative mass spectra of AGs multiacylated by Eis. (A) Mono-, di-, and tri-*n*-propionyl-NEO (m/z $[M+H]^+$ 671.35, 727.40, and 783.60, respectively) generated by reaction of NEO, Eis, and ProCoA. (B) Mono-crotonyl-SIS (m/z $[M+H]^+$ 516.25) generated by reaction of SIS, Eis, and CroCoA. (C) Mono- and di-malonyl-NET (m/z $[M+H]^+$ 562.20 and 648.20, respectively) generated by reaction of NET, Eis, and MalCoA.

mM). Reactions were initiated by the addition of CoA derivatives and were carried out at least in duplicate at 25°C. The reactions were monitored as described above, taking measurements every 15 s for 15 min. The kinetic parameters K_m and k_{cat} were determined from Lineweaver-Burk plots (Table 1).

TLC assays. The eluent system used for AMK reactions was 5:2 MeOH-NH₄OH (~25% in H₂O). The eluent system utilized for NEA reactions was 3:0.8 MeOH-NH₄OH (~25% in H₂O). The eluent system employed for NET reactions was 12:1 MeOH-NH₄OH (~25% in H₂O). AGs were visualized on silica gel 60 F₂₅₄ thin-layer chromatography (TLC) plates (Merck) by using a cerium-molybdate stain composed of (NH₄)₂Ce(NO₃)₆ (5 g), (NH₄)₆Mo₇O₂₄ · 4H₂O (120 g) in 10% H₂SO₄ (1 liter). The observed R_f values are reported in Table S1 in the supplemental material. The exact reaction conditions are reported below.

TABLE 1 Kinetic parameters determined for Eis and acyl-CoA derivatives with various AGs

CoA analog	AG	Value (mean \pm SD) of:		
		K_m (μ M)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
Acetyl-CoA	AMK	7.88 \pm 1.35	1.14 \pm 0.30	2,411 \pm 757
	HYG	176.62 \pm 6.42	16.26 \pm 0.48	1,534 \pm 72
	KAN	9.44 \pm 1.20	5.56 \pm 0.36	9,816 \pm 1,400
	NEA	47.30 \pm 1.46	4.50 \pm 0.36	1,586 \pm 136
	NEO	24.44 \pm 0.68	6.54 \pm 0.18	4,460 \pm 175
	NET	62.95 \pm 2.44	14.82 \pm 1.02	3,923 \pm 310
	PAR	45.50 \pm 5.52	3.24 \pm 0.78	1,187 \pm 320
	RIB	22.18 \pm 1.72	3.54 \pm 0.06	2,660 \pm 211
	SIS	69.41 \pm 0.80	4.20 \pm 0.12	1,009 \pm 31
	TOB	47.04 \pm 1.31	16.92 \pm 4.92	5,995 \pm 1,751
<i>n</i> -Propionyl-CoA	AMK	53.14 \pm 6.18	1.14 \pm 0.30	358 \pm 103
	HYG	30.84 \pm 0.62	1.80 \pm 0.01	973 \pm 20
	KAN	55.66 \pm 13.28	0.48 \pm 0.06	144 \pm 39
	NEA	55.53 \pm 6.08	0.60 \pm 0.06	180 \pm 28
	NEO	53.22 \pm 0.14	1.98 \pm 0.12	620 \pm 38
	NET	31.24 \pm 3.76	3.23 \pm 0.02	1,723 \pm 208
	PAR	25.12 \pm 2.62	0.48 \pm 0.06	318 \pm 52
	RIB	66.76 \pm 3.61	1.32 \pm 0.30	330 \pm 77
	SIS	105.79 \pm 4.79	6.42 \pm 1.50	1,011 \pm 241
	TOB	74.49 \pm 7.78	6.36 \pm 0.24	1,423 \pm 158
Crotonyl-CoA	NEO	88.86 \pm 7.40	0.33 \pm 0.04	62 \pm 9
Malonyl-CoA	NEO	81.49 \pm 5.07	1.51 \pm 0.02	309 \pm 20

(i) **Control TLCs for mono-*n*-propionylated AGs at the 2', 3-, or 6'-position.** Reactions (10- μ l mixtures) were performed at RT in morpholineethanesulfonic acid (MES) buffer [50 mM, pH 6.6, for AAC(3)-IV and AAC(6')] or in potassium phosphate buffer [100 mM, pH 7.0, for AAC(2')-Ic] with ProCoA (0.96 mM, 1.2 eq), AG (0.8 mM, 1 eq), and AAC enzyme (10 μ M). After overnight incubation, aliquots (5 μ l) of the reaction mixtures were loaded onto a TLC plate and eluted using the solvent system described above.

(ii) **Control TLCs for di-*n*-propionylated NEA by sequential enzymatic reactions.** Reactions (10- μ l mixtures) were performed at RT in MES buffer (50 mM, pH 6.6, adjusted at RT) with ProCoA (1.92 mM, 2.4 eq), NEA (0.8 mM, 1 eq), and AAC(6') or AAC(3)-IV (10 μ M). After overnight incubation, the second AAC enzyme [AAC(2')-Ic or AAC(3)-IV] (10 μ M) was added to the reaction mixture. After an additional 24 h of incubation, aliquots (5 μ l) of each di-*n*-propionylation reaction mixture were loaded onto a TLC plate and eluted using the solvent system described above.

(iii) **TLCs for *n*-propionylation of AMK and NET by Eis.** Reactions (20- μ l mixture) were performed at RT in Tris-HCl buffer (50 mM, pH 8.0, adjusted at RT) with ProCoA (2.5 mM, 5 eq), AG (0.5 mM, 1 eq), and Eis (10 μ M). After overnight incubation, aliquots (5 μ l) of the reaction mixture were loaded onto a TLC plate and eluted using the solvent system described above (see Fig. S5 in the supplemental material).

(iv) **TLC time course for *n*-propionylation of NEA by Eis.** Reactions (30- μ l mixtures) were performed at RT in Tris-HCl buffer (50 mM, pH 8.0, adjusted at RT) with ProCoA (2.5 mM, 5 eq), NEA (0.5 mM, 1 eq), and Eis (5 μ M). Aliquots (4 μ l) were loaded and run on a TLC plate after incubation times of 0, 1, 5, 10, 30, 120 min, and overnight using the solvent system described above (Fig. 2).

Monitoring by LC-MS of sequential acylations by Eis using ProCoA followed by AcCoA. Reaction mixtures (30 μ l) containing AG (0.67 mM, 1 eq), ProCoA (3.35 mM, 5 eq), Tris-HCl (50 mM, pH 8.0, adjusted at RT), and Eis (5 μ M) were incubated at RT for 48 h prior to the addition of AcCoA (3.35 mM, 5 eq) and an additional portion of Eis (5 μ M), which brought the total volume of the reaction mixture to 50 μ l. After another 48 h of incubation, the Eis protein was precipitated by the addition of ice-cold MeOH (50 μ l) to the reaction mixture, which was then kept at -20°C

for at least 20 min. The precipitated protein was removed by centrifugation (13,000 rpm, RT, 10 min). An aliquot of the supernatant (20 μ l) was analyzed by LC-MS in the positive mode by using 0.1% (vol/vol) formic acid in H_2O (see Fig. S6 and Table S3 in the supplemental material).

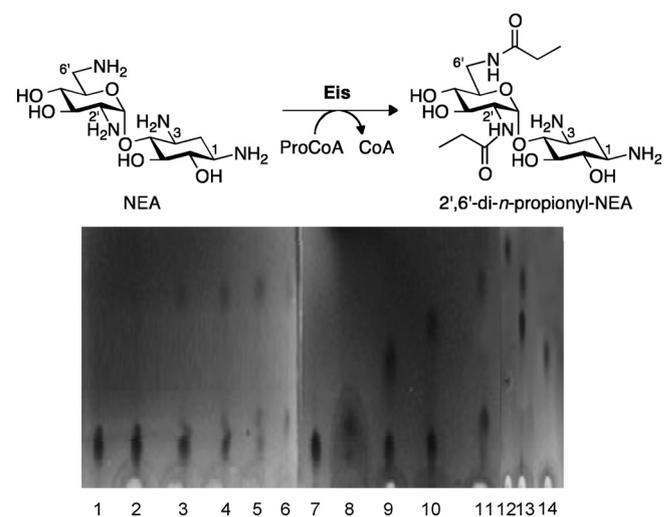


FIG 2 TLC time course showing the 2'-mono- and 2',6'-di-*n*-propionylated NEA products generated by Eis using 5 equivalents of ProCoA. Control reactions for mono- and di-*n*-propionylation were done using AAC(2')-Ic, AAC(3)-IV, and AAC(6') individually or sequentially. Lane 1, NEA (R_f 0.11); lane 2, NEA + ProCoA + Eis (1 min) (R_f 0.11, 0.13); lane 3, NEA + ProCoA + Eis (5 min) (R_f 0.11, 0.13, 0.41); lane 4, NEA + ProCoA + Eis (10 min) (R_f 0.11, 0.13, 0.41); lane 5, NEA + ProCoA + Eis (30 min) (R_f 0.11, 0.13, 0.41); lane 6, NEA + ProCoA + Eis (2 h) (R_f 0.11, 0.13, 0.41); lane 7, NEA (R_f 0.11); lane 8, 2'-*n*-propionyl-NEA (R_f 0.13); lane 9, 3-*n*-propionyl-NEA (R_f 0.24); lane 10, 6'-*n*-propionyl-NEA (R_f 0.33); lane 11, NEA + ProCoA + Eis (O/N) (R_f 0.41); lane 12, 6',3-di-*n*-propionyl-NEA (R_f 0.53); lane 13, 6',2'-di-*n*-propionyl-NEA (R_f 0.41); lane 14, 3,2'-di-*n*-propionyl-NEA (R_f 0.26).

Monitoring by LC-MS of competition of modification reactions by Eis using ProCoA and AcCoA simultaneously. Reaction mixtures (50 μ l) containing AG (0.67 mM, 1 eq), AcCoA (3.35 mM, 5 eq), ProCoA (3.35 mM, 5 eq), Tris-HCl (50 mM, pH 8.0, adjusted at RT), and Eis (10 μ M) were incubated at RT for 48 h, after which they were processed and analyzed by LC-MS as described above for the sequential acylations (see Fig. S7 and Table S3 in the supplemental material).

RESULTS

Acyl-CoA cosubstrate tolerance of Eis. We previously reported that Eis is an AAC capable of multiacetylation of a variety of AG scaffolds by using AcCoA as a cosubstrate (10). Here, in order to determine the cosubstrate profile of Eis, we tested 15 additional acyl-CoA derivatives with the five Eis AG substrates with the best catalytic transfer efficiency (10). By UV-Vis assay, we established that only three of the acyl-CoA derivatives tested, CroCoA, MalCoA, and ProCoA, displayed any significant reactivity with an AG in the presence of Eis. To gain a better understanding of the catalytic efficiency of Eis toward its accepted cosubstrates, we first determined the steady-state kinetic parameters for AcCoA and ProCoA with all 10 AGs (AMK, HYG, KAN, NEA, NEO, NET, PAR, RIB, SIS, and TOB) (Table 1). Briefly, for AcCoA, the K_m values ranged from $7.88 \pm 1.35 \mu\text{M}$ (mean \pm standard deviation) for AMK to $176.62 \pm 6.42 \mu\text{M}$ for HYG, representing a 22-fold range. The catalytic turnover constants varied in a 15-fold range, with AMK displaying the lowest k_{cat} value ($1.14 \pm 0.30 \text{ min}^{-1}$) and TOB exhibiting the highest k_{cat} value ($16.92 \pm 4.92 \text{ min}^{-1}$). Overall, the catalytic efficiencies (k_{cat}/K_m) for AcCoA varied in a 10-fold range, with SIS displaying the lowest catalytic efficiency ($1,009 \pm 31 \text{ M}^{-1} \text{ s}^{-1}$) and KAN the highest ($9,816 \pm 1,400 \text{ M}^{-1} \text{ s}^{-1}$). For ProCoA, the K_m values ranged from $25.12 \pm 2.62 \mu\text{M}$ for PAR to $105.79 \pm 4.79 \mu\text{M}$ for SIS, a 4.2-fold range. Interestingly, the higher variability of the K_m values for AcCoA is due solely to the two AGs that are used against TB in clinical practice, KAN and AMK, which display significantly lower μM K_m values than do the other AGs with AcCoA but not with ProCoA. The k_{cat} values varied in a 13-fold range, with KAN exhibiting the slowest catalytic turnover ($0.48 \pm 0.06 \text{ min}^{-1}$) and SIS the fastest ($6.42 \pm 1.50 \text{ min}^{-1}$). Generally, the catalytic efficiencies for ProCoA spanned an 8.5-fold range, with KAN exhibiting the lowest k_{cat}/K_m value ($144 \pm 39 \text{ M}^{-1} \text{ s}^{-1}$) and NET the highest ($1,723 \pm 208 \text{ M}^{-1} \text{ s}^{-1}$). The order of AGs ranked by either their K_m or k_{cat} values was different for ProCoA than for AcCoA. We next determined the steady-state kinetic parameters for MalCoA and CroCoA using NEO as a substrate. MalCoA had a K_m of $81.49 \pm 5.07 \mu\text{M}$ and a k_{cat} of $1.51 \pm 0.02 \text{ min}^{-1}$, resulting in a catalytic efficiency of $309 \pm 20 \text{ M}^{-1} \text{ s}^{-1}$. CroCoA displayed a K_m of $88.86 \pm 7.40 \mu\text{M}$ and a k_{cat} of $0.33 \pm 0.04 \text{ min}^{-1}$, yielding an efficiency of $62 \pm 9 \text{ M}^{-1} \text{ s}^{-1}$.

Multiplicity of AG acylation by Eis. We recently reported that Eis can acetylate between two to four amines on the same AG, depending on its scaffold (10). To investigate the maximum number of sites that can be *n*-propionylated, crotonylated, and malonylated by Eis, we monitored by mass spectrometry the reactions of 10 AGs (AMK, HYG, KAN, NEA, NEO, NET, PAR, RIB, SIS, and TOB) with ProCoA, CroCoA, and MalCoA, respectively (Table 2 and Fig. 1; also see Table S2 and Fig. S2 to S4 in the supplemental material). When using ProCoA as the cosubstrate, we observed that the 10 AGs tested were modified, but the number of acylations was generally different from the number of acetylations. HYG, NET, PAR, and SIS were mono-*n*-propionylated,

TABLE 2 Comparison of the numbers of acylations for reactions of Eis with various acyl-CoAs and AGs

AG	No. of acylations ^a for indicated reaction			
	Acetyl ^b (CH ₃)	<i>n</i> -Propionyl (CH ₂ CH ₃)	Crotonyl (CH=CHCH ₃)	Malonyl (CH ₂ CO ₂ H)
AMK	Tri	Di	Mono	—
HYG	Di	Mono	—	—
KAN	Di	Di	Mono	—
NEA	Tri	Di	Mono	—
NEO	Tri	Tri	Mono	Mono
NET	Di	Mono	Mono	Di
PAR	Di	Mono	Mono	Mono
RIB	Tri	Di	—	—
SIS	Tri	Mono	Mono	Mono
TOB	Tetra	Di	Mono	—

^a Mono, Di, Tri, and Tetra indicate single, double, triple, and quadruple modifications, respectively; a dash indicates no modification.

^b Data are from reference 10.

AMK, KAN, NEA, RIB, and TOB became di-*n*-propionylated, and NEO turned out to be tri-*n*-propionylated. When utilizing the CroCoA cosubstrate, we found that Eis transferred a single propionyl group to eight AG scaffolds (AMK, KAN, NEA, NEO, NET, PAR, SIS, and TOB). We determined that MalCoA modified only four AGs, of which three were monomalonylated (NEO, PAR, and SIS) and one dimalonylated (NET).

Order of AG *n*-propionylation by Eis. Earlier, we demonstrated that Eis triacetylates NEA in a sequential manner by first modifying the 2' position, followed by the 6' and then 1 positions (10). Here, we showed that Eis can di-*n*-propionylate NEA. To determine whether the order of *n*-propionylation of NEA by Eis is consistent with that of the first two acetylations of this AG scaffold by this enzyme, we explored by TLC assay the regiospecificity and order of NEA *n*-propionylation by Eis (Fig. 2). By comparing the R_f values of *n*-propionylated Eis products formed over time to mono- and di-*n*-propionylated NEA standards obtained by using, individually or sequentially, AAC enzymes that catalyze a single acylation reaction [AAC(6'), AAC(3)-IV, and AAC(2')-Ic], we established that the order of *n*-propionylation of NEA was similar to that of acetylation, with the 2' position being modified first and the 6' position second. Using a similar TLC approach, we also performed a preliminary investigation of the order of *n*-propionylation of two other AGs, AMK and NET (see Fig. S5 in the supplemental material). AMK appeared to be *n*-propionylated at the 6' position and at a second site that could not be identified by TLC, as AMK could not be diacetylated using AAC(6'), AAC(3)-IV, and AAC(2')-Ic sequentially or simultaneously (see Fig. S5A in the supplemental material). By comparing the retention factor of the product of the reaction of NET with ProCoA and Eis ($R_f = 0.36$) to that of a 6'-*n*-propionyl-NET standard ($R_f = 0.36$), we determined that 6'-*n*-propionyl-NET was the product of the enzymatic reaction (see Fig. S5B). These results indicate that the 6' position is a common site of acylation of AGs by Eis.

Sequential modifications of AGs by Eis. By close inspection of the data presented in Table 2, we observed that for almost all AGs studied, the number of sites of *n*-propionylation was lower than the number of sites of acetylation. To examine whether the *n*-propionylated AGs could be subsequently acetylated, we first incubated AGs with Eis and an excess of ProCoA, and after comple-

TABLE 3 Comparison of the number of acylations for sequential or competition reactions of Eis with different acyl-CoAs and AGs

AG	No. of acylations/propionylations ^a for indicated reaction			
	Acetyl ^b	<i>n</i> -Propionyl	Pro → Ac ^c	Ac + Pro ^d
AMK	Tri	Di	Mono-Ac-di-Pro	Di-Ac tri-Ac
HYG	Di	Mono	Mono-Pro	Mono-Ac mono-Ac-mono-Pro
KAN	Di	Di	Di-Pro	Di-Ac
NEA	Tri	Di	Mono-Ac-di-Pro	Tri-Ac di-Ac-mono-Pro
NEO	Tri	Tri	Tri-Pro	Tri-Ac tri-Pro
NET	Di	Mono	Mono-Pro	Mono-Ac di-Ac
PAR	Di	Mono	Mono-Ac-mono-Pro	Di-Ac
RIB	Tri	Di	Di-Pro	Di-Ac
SIS	Tri	Mono	Mono-Pro	Mono-Ac di-Ac mono-Ac-mono-Pro
TOB	Tetra	Di	Mono-Ac-di-Pro di-Ac-di-Pro	Di-Ac tri-Ac tetra-Ac

^a Mono, Di, Tri, and Tetra indicate single, double, triple, and quadruple modifications, respectively.

^b Data are from reference 10.

^c ProCoA reactions were followed by incubation with AcCoA.

^d AcCoA and ProCoA were incubated with Eis and AG in a 1:1 ratio.

tion of the *n*-propionylation reactions, we added an excess of AcCoA. By mass spectrometry analysis, we established that the results of these sequential modification studies could be broken down into three scenarios (Table 3; also see Fig. S6 in the supplemental material) after complete *n*-propionylation, as follows. (i) Some AGs, intriguingly, were further acetylated to reach the number of modifications presented in the column for acetylation reactions in Table 3. This was the case for AMK, NEA, PAR, and TOB, whose final products were mono-Ac-di-Pro-AMK, mono-Ac-di-Pro-NEA, mono-Ac-mono-Pro-PAR, and di-Ac-di-Pro-TOB, respectively. (ii) Some AGs were not further derivatized, which was observed when the number of *n*-propionylations reached the reported number of acetylations. This was the scenario followed by KAN and NEO, where only di-Pro-KAN and tri-Pro-NEO, respectively, were produced. (iii) The other AGs were not further modified even though the amount of acetylation reported was larger than the amount of *n*-propionylation. This was the case for HYG, NET, RIB, and SIS, where mono-Pro-HYG, mono-Pro-NET, di-Pro-RIB, and mono-Pro-SIS were formed, respectively.

Competition assays using AcCoA and ProCoA simultaneously for AG modifications by Eis. As both AcCoA and ProCoA are found in abundance inside bacterial cells, to corroborate our steady-state kinetic analysis studies, we performed cosubstrate competition assays where AGs were incubated in the presence of Eis and a 1:1 mixture of AcCoA and ProCoA (Table 3; also see Fig. S7 in the supplemental material). As expected, in all cases (AMK, KAN, NET, PAR, and RIB) where the catalytic efficiencies for transfer of an acetyl group were far superior to those for transfer of an *n*-propionyl moiety, multiacetylation occurred almost exclusively. Two exceptions to this pattern were observed with NEA and NEO. For NEA, with k_{cat}/K_m values of $1,586 \pm 136 \text{ M}^{-1} \text{ s}^{-1}$ for AcCoA and $180 \pm 32 \text{ M}^{-1} \text{ s}^{-1}$ for ProCoA, one would have expected multiacetylation to be almost exclusive. However, di-Ac-mono-Pro-NEA was detected by mass spectrometry when NEA was reacted with a 1:1 mixture of AcCoA and ProCoA. For NEO, with k_{cat}/K_m values of $4,460 \pm 39 \text{ M}^{-1} \text{ s}^{-1}$ for AcCoA and $620 \pm 38 \text{ M}^{-1} \text{ s}^{-1}$ for ProCoA, one would have expected multiacetylation to dominate. However, both tri-Ac-NEO and tri-Pro-NEO were detected by mass spectrometry. As predicted for HYG and SIS, which had similar catalytic efficiencies for AcCoA ($1,534 \pm 72$

$\text{M}^{-1} \text{ s}^{-1}$ for HYG and $1,009 \pm 31 \text{ M}^{-1} \text{ s}^{-1}$ for SIS) and ProCoA ($973 \pm 20 \text{ M}^{-1} \text{ s}^{-1}$ for HYG and $1,011 \pm 241 \text{ M}^{-1} \text{ s}^{-1}$ for SIS), mono-Ac-mono-Pro-AG were detected by mass spectrometry.

DISCUSSION

Many enzymes display natural and/or engineered substrate and/or cosubstrate promiscuity/tolerance. A few examples of naturally catalytically promiscuous enzymes include hydrolases (5), the enzyme macrophomate synthase (18), the strictosidine synthase family of enzymes (1), ketoreductase enzymes involved in polyketide biosynthesis (33), the acyltransferase CouN7 (16), and AACs (20, 38). Cytochrome P450 monooxygenases have been engineered to expand their cosubstrate promiscuity (29). In addition, the broad substrate profile of antibiotic-inactivating enzymes has often resulted from mutations in the genes encoding the enzymes, as exemplified by β -lactamases (32). We have demonstrated that Eis is an AAC capable of multiacetylating a large number of AGs (10). Here, we performed a detailed study to establish the acyl-CoA cosubstrate tolerance of Eis in order to gain insight into its multiacetylation mechanism.

We first examined the cosubstrate profile of Eis using 16 acyl-CoAs with five AGs. In doing so, we observed that in contrast to other AACs that exhibit broad cosubstrate promiscuity, Eis catalyzed transfer only from a small set of the acyl-CoA derivatives. In addition to the natural cosubstrate AcCoA, only CroCoA, Mal-CoA, and ProCoA were found to act as cosubstrates of Eis. To rationalize the limited cosubstrate tolerance of Eis, we closely examined its structure and those of other AACs with bound cosubstrate, including AAC(3)s (23, 27, 43), AAC(2')-Ic from *M. tuberculosis* (39), and AAC(6')s (3, 4, 31, 40, 41, 44). Even though the AG-binding pocket of Eis is larger, the access to it is more limited than in other AACs due to the presence of the central GNAT region, absent in other AACs, whose surface contributes to the substrate-binding pocket (see Fig. S8A in the supplemental material) (10). This suggests that in AACs other than Eis, the substrate/cosubstrate promiscuity is due to the relatively accessible active sites of these enzymes. For example, in AAC(6')-Ii from *Enterococcus faecium* (3), the AG-binding pocket is open to entry both in the direction orthogonal to the Ppant arm of AcCoA (from the side of the viewer in Fig. S8B) and in the direction along the Ppant

arm toward the thiol moiety (from the left-hand side in Fig. S8B), whereas for Eis, access is possible only in the orthogonal direction. Therefore, it is likely that the larger acyl groups of cosubstrates block the access of the AG to the binding pocket in Eis. It is important to note that most AMEs were acquired to reduce the toxicity of AGs in AG-producing organisms (12), while Eis evolved through selective pressure to modify AGs (45). Interestingly, while Eis could not transfer the acyl moieties of CoA derivatives with bulkier (benzoyl, bromothiophene-2-carbonyl, and fluoro-picolinyl) or longer (butyryl and glutaryl) acyl groups that AAC(3)-IV and AAC(6')/APH(2'') could transfer to a variety of AGs, Eis was found to readily transfer the crotonyl moiety to eight AGs that the other two AACs could not. With the exception of KAN and NEO, for which the numbers of sites *n*-propionylated and acetylated were identical, we observed that in every other case, the number of sites acylated was less than the number of sites acetylated (Table 2). Not surprisingly, while the smaller and less rigid *n*-propionyl group could be transferred to more than one position on 6 of the 10 AGs tested, the larger malonyl and the less-flexible crotonyl moieties were generally only transferred to one amine on some of the AGs tested.

We next determined the kinetic parameters (K_m and k_{cat}) for Eis and acyl-CoAs with a variety of AGs and established that for the majority of the AGs tested, the binding affinity of ProCoA to Eis was less than that of AcCoA (Table 1). We first corroborated our kinetic data by exploring the sequential modifications of AGs by Eis, using first an excess of ProCoA, followed by an excess of AcCoA (Table 3; also see Fig. S6 in the supplemental material). In the cases where the number of *n*-propionylated sites was smaller than the number of acetylated sites and where the catalytic efficiency (k_{cat}/K_m) of transfer of the acyl group was much lower for ProCoA than for AcCoA, we observed that after maximum *n*-propionylation, the AGs were further acetylated to reach the number of modified sites equal to the maximum number of acetylations observed previously. With RIB being the exception, as it did not get further acetylated after di-*n*-propionylation, this was the case for AMK, NEA, PAR, and TOB. AMK, with k_{cat}/K_m values of $2,411 \pm 757 \text{ M}^{-1} \text{ s}^{-1}$ and $358 \pm 103 \text{ M}^{-1} \text{ s}^{-1}$ for AcCoA and ProCoA, respectively, was transformed into mono-Ac-di-Pro-AMK. NEA, with k_{cat}/K_m values of $1,586 \pm 136 \text{ M}^{-1} \text{ s}^{-1}$ and $180 \pm 28 \text{ M}^{-1} \text{ s}^{-1}$ for AcCoA and ProCoA, respectively, was converted into mono-Ac-di-Pro-NEA. PAR, with k_{cat}/K_m values of $1,187 \pm 320 \text{ M}^{-1} \text{ s}^{-1}$ and $318 \pm 52 \text{ M}^{-1} \text{ s}^{-1}$ for AcCoA and ProCoA, respectively, was modified into mono-Ac-mono-Pro-PAR. TOB, with k_{cat}/K_m values of $5,995 \pm 1,751 \text{ M}^{-1} \text{ s}^{-1}$ and $1,423 \pm 158 \text{ M}^{-1} \text{ s}^{-1}$ for AcCoA and ProCoA, respectively, was transformed into di-Ac-di-Pro-TOB. In the cases of HYG, NET, and SIS, where the numbers of sites *n*-propionylated were lower than the numbers of positions acetylated and the catalytic efficiency (k_{cat}/K_m) of transfer of the acyl group was similar for ProCoA and AcCoA, we observed that the AGs were not further acetylated after complete *n*-propionylation. Finally, as expected, regardless of the catalytic efficiency of acyl transfers, we observed that KAN and NEO were not further acetylated by Eis after di- and tri-*n*-propionylation, as the level of number of sites acetylated and *n*-propionylated were equal. We conclude that modification with a large acyl group must, at least in some cases, prevent binding of the acylated AG to allow further acylation or acetylation for steric reasons.

We also corroborated our steady-state kinetic analysis studies and gained valuable knowledge about Eis by investigating the competition between *n*-propionylation and acetylation of AGs by

this enzyme (Table 3; also see Fig. S7 in the supplemental material). In agreement with their much higher k_{cat}/K_m values for AcCoA, we observed that AMK, KAN, NET, PAR, RIB, and TOB were solely acetylated when reacted with a 1:1 mixture of ProCoA and AcCoA. The aforementioned AGs have a higher catalytic efficiency, lower K_m value, higher catalytic turnover rate, or a combination of these properties, which all can explain the dominating effect of AcCoA in the competition assays. Interestingly in the case of RIB, we observed only diacetylation of the AG in the presence of a 1:1 mixture of AcCoA and ProCoA, potentially suggesting that the presence of ProCoA inhibits the transfer of the third acetyl group to di-Ac-RIB and *n*-propionylation cannot occur at the third acylation site. Interestingly, in the case of NEO, where the k_{cat}/K_m value for AcCoA ($4,460 \pm 175 \text{ M}^{-1} \text{ s}^{-1}$) was much larger than that for ProCoA ($620 \pm 38 \text{ M}^{-1} \text{ s}^{-1}$), one would have expected multiacetylation to be dominant. However, both tri-Ac-NEO and tri-Pro-NEO were generated when NEO was reacted with a 1:1 mixture of AcCoA and ProCoA. This implies that after the first modification with a particular cosubstrate, only the second and third modifications by a different cosubstrate are strongly disfavored. In addition, the modifications do not follow the same rank order of catalytic efficiency. A plausible model for this effect is that after a NEO molecule is modified once by either AcCoA or ProCoA, the modified AG does not leave the enzyme active site but, rather, the second and third modifications occur before the singly modified AG can dissociate from the binding site, resulting in the homogeneity of the modifications of NEO. Finally, as expected, in the case of HYG and SIS, where the k_{cat}/K_m values for AcCoA ($1,534 \pm 72 \text{ M}^{-1} \text{ s}^{-1}$ for HYG and $1,009 \pm 31 \text{ M}^{-1} \text{ s}^{-1}$ for SIS) and ProCoA ($973 \pm 20 \text{ M}^{-1} \text{ s}^{-1}$ for HYG and $1,011 \pm 241 \text{ M}^{-1} \text{ s}^{-1}$ for SIS) were very similar, both acetylation and *n*-propionylation occurred. The reaction of a 1:1 AcCoA-ProCoA mixture with HYG in the presence of Eis resulted in mono-Ac-mono-Pro-HYG. From our sequential experiments, we established that acetylation of HYG must occur first in order for mono-Ac-mono-Pro-HYG to be formed. We also previously reported that HYG can be diacetylated (10). Therefore, it can be hypothesized that mono-Ac-HYG is a better substrate for *n*-propionylation than for a second acetylation. We can also draw a similar conclusion from the results obtained with SIS. The reaction of SIS with a 1:1 AcCoA-ProCoA mixture in the presence of Eis resulted in mono-Ac-SIS, di-Ac-SIS, and mono-Ac-mono-Pro-SIS. Since we know that mono-*n*-propionylation of SIS results in an unreactive species in our sequential experiments, we can conclude that acetylation must occur before *n*-propionylation in order to generate mono-Ac-mono-Pro-SIS. Based on the competition assay (Table 3) and the kinetic parameters determined (Table 1), we also conclude that mono-Ac-SIS has a similar propensity to become acetylated the second time or to become *n*-propionylated.

In summary, we have presented evidence that, albeit more limited than that of other AACs, Eis displays some cosubstrate tolerance, accepting AcCoA, ProCoA, CroCoA, and MalCoA for the multiacylation of AGs. We have established the number of sites acylated by these cosubstrates on a variety of AG scaffolds. We have demonstrated that the order of multiacylation for NEA is identical to the order of multiacetylation. We have shown that after complete *n*-propionylation, further acetylations can be allowed up to the level of multiacetylation allowed by Eis. Finally, by steady-state kinetic assays and acylation competition assays, we

have gained insight into the mechanism of multiacylation of AGs by Eis.

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