FUNCTIONAL AND KINETIC ANALYSIS OF THE PHOSPHOTRANSFERASE CapP
CONFERRING SELECTIVE SELF-RESISTANCE TO CAPURAMYCIN ANTIBiotics

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Capuramycin-related compounds, including A-500359s and A-503083s, are nucleoside antibiotics that inhibit the enzyme bacterial translocase I (MraY) involved in peptidoglycan cell wall biosynthesis. Within the biosynthetic gene cluster for the A-500359s exists a gene encoding a putative aminoglycoside 3-phosphotransferase (APH) that was previously demonstrated to be highly expressed during the production of A-500359s and confers selective resistance to capuramycins when expressed in heterologous hosts. A similar gene (capP) was identified within the biosynthetic gene cluster for the A-503083s, and CapP is now shown to similarly confer selective resistance to capuramycins. Recombinant CapP was produced and purified from Escherichia coli and the function of CapP is established as an ATP-dependent capuramycin phosphotransferase that regio-specifically transfers the γ-phosphate to the 3″-hydroxyl of the unsaturated hexuronic acid moiety of A-503083 B. Kinetic analysis with the three major A-503083 congeners suggests CapP preferentially phosphorylates A-503083s containing an aminocaprolactam moiety attached to the hexuronic acid, and bi-substrate kinetic analysis was consistent with CapP employing a sequential kinetic mechanism similar to most known APHs. The purified CapP product lost its antibiotic activity against Mycobacterium smegmatis, and this loss in bioactivity is primarily due to a 272-fold increase in the IC50 in the MraY-catalyzed reaction. The results establish CapP-mediated phosphorylation as a mechanism of resistance to capuramycins, and now sets the stage to explore this strategy of resistance as a potential mechanism inherent to pathogens and provides the impetus for preparing second generation analogues as a preemptive strike to such resistance strategies.

Infectious and parasitic diseases are the second leading cause of death worldwide, and this includes more than 1.6 million deaths by tuberculosis (TB) in 2006 (1). Alarmingly, nearly 5% of all of the new cases of TB during this same year were caused by Mycobacterium tuberculosis—the primary causative agent of TB—that had resistance to at least one of the commonly used antibiotics to treat TB (2). To compound this problematic emergence of drug resistance, the discovery and development of new antibiotics has continued to decline with only a few new classes of antibiotics introduced into the clinic within the past few decades (3, 4). Thus, there is a great need for new antibiotics with novel modes of action and unique structures to combat multiple drug resistant (MDR) pathogens such as M. tuberculosis.

Capuramycin was discovered from Streptomyces griseus 446-S3 based on gross antibacterial activity (5), and analogues termed A-500359s from Streptomyces griseus SANK 60196 (6-8) and A-503083s from Streptomyces sp. SANK 60196.
feeding 2-aminoethyl-deaminocaprolactam analogues were isolated upon 500359 D (6). Subsequently, a variety of deshydroxy analogue of A-500359 B, termed A-G and A-500359 C, respectively, and the analogues of A-500359 A and B, termed A-500359 the second major congener produced from Isolation and structural elucidation of A-500359 B, acid, and an aminocaprolactam moiety (17). The structure of capuramycin was initially solved in 1988 to reveal three distinct moieties, a 5'-solved in 1988 to reveal three distinct moieties, a 5'-aminocaprolactam moiety and is the major congener produced (Fig. 1) (6). Four other A-500359 congeners were named to correspond with the respective A-500359s, for example A-503083 A and A-503083 B shown in Fig. 1. Interestingly, in contrast to A-500359 E and F, the deaminocaprolactam analogues A-503083 E and F were isolated as the second and third major congeners using standard fermentation conditions without the addition of an aspartokinase inhibitor (9).

The biosynthetic gene cluster for A-500359s was subsequently discovered using a specific screen aimed at identifying bacterial translocase I (MraY) inhibitors. MraY initiates the lipid cycle of peptidoglycan cell wall biosynthesis, a pathway that is essential for the survival of all bacteria (10). MraY catalyzes the transfer of phospho-N-acetylmuramic acid-pentapeptide from UDP-N-acetylmuramic acid-pentapeptide to undecaprenyl phosphate, releasing UMP to generate undecaprenyl-disphospho-N-acetylmuramic acid-pentapeptide, or Lipid I. Crude preparations of MraY from solubilized membrane fractions have been sufficient in validating the enzyme activity, determining the preliminary biochemical characteristics such as Mg<sup>2+</sup> dependence, and elucidating apparent kinetic constants (11-13). Recently, the Bacillus subtilis MraY enzyme has been isolated to apparent homogeneity and shown to retain some activity, and the active site was partially mapped by site-directed mutagenesis (14). Despite the difficulties associated with the protein preparation, several unique classes of antibiotics including the capuramycins have now been shown to competitively or noncompetitively inhibit MraY (11, 15). The proven success of cell wall biosynthesis inhibitors in the clinic suggest a potential utility for capuramycins and other MraY inhibitors as antibiotics (16).

The structure of capuramycin was initially solved in 1988 to reveal three distinct moieties, a 5'-C-carbamoyluridine, an unsaturated hexuronic acid, and an aminocaprolactam moiety (17). Isolation and structural elucidation of A-500359 B, the second major congener produced from S. griseus SANK 60196, revealed an identical structure to that originally reported for capuramycin (Fig. 1) (6). Four other A-500359 congeners were also structurally assigned, including A-500359 A, which contains an additional C-methyl group within the aminocaprolactam moiety and is the major congener produced (Fig. 1), the 3'-O-desmethyl analogues of A-500359 A and B, termed A-500359 G and A-500359 C, respectively, and the deshydroxy analogue of A-500359 B, termed A-500359 D (6). Subsequently, a variety of deaminocaprolactam analogues were isolated upon feeding 2-aminoethyl-L-cysteine, a specific inhibitor of aspartokinase involved in lysine biosynthesis in actinomycetes, including the free acid A-500359 F and the methyl ester A-500359 E (8). Following the discovery of A-500359s, four capuramycin-type antibiotics named A-503083s were isolated from a different Streptomyces strain, and structural analysis revealed that, unlike the A-500359s, the A-503083s contain an additional 2'-O-carbamoyl group that slightly decreases the MraY inhibitory activity by 2- to 8-fold (9). The four congeners were named to correspond with the respective A-500359s, for example A-503083 A and A-503083 B shown in Fig. 1. Interestingly, in contrast to A-500359 E and F, the deaminocaprolactam analogues A-503083 E and F were isolated as the second and third major congeners using standard fermentation conditions without the addition of an aspartokinase inhibitor (9).

The biosynthetic gene cluster for A-500359s was cloned and sequenced to reveal a gene product (ORF 21) within the locus that has sequence similarity to aminoglycoside phosphotransferases (APHs) (18). APHs catalyze the regio-specific phosphorylation of a variety of aminoglycosides and are often found in bacterial pathogens such as species of Enterobacteriaceae and Staphylococcus that do not produce aminoglycosides, but are also found encoded within the biosynthetic gene cluster of a producing organism of a given aminoglycoside (19). Thus, it was hypothesized that expression of orf21 provides S. griseus SANK 60196 a mechanism of self-resistance to capuramycins by modification of the antibiotic upon production. As expected, heterologous expression of orf21 in Streptomyces albus and Escherichia coli ΔtolC, two strains that are normally sensitive to capuramycin antibiotics, yielded strains that were resistant to A-500359 B (18). Furthermore, heterologous expression of orf21 did not affect the sensitivity of these strains to representative aminoglycosides suggesting the resistance is specific for capuramycins. However, the exact mechanism of resistance by ORF21 has hitherto only been inferred based on sequence analysis.

The biosynthetic gene cluster for A-503083s was recently cloned and characterized, revealing a gene product CapP with high sequence similarity to ORF21 (20). Herein we describe the functional and kinetic studies of CapP to demonstrate that this enzyme is an ATP-dependent capuramycin 3’-phosphotransferase, and phosphorylation not only
results in a complete loss of antibiotic activity but also significantly reduces the capuramycins ability to inhibit the MraY-catalyzed reaction, thus suggesting the 3′-hydroxyl group is critical for bioactivity. The presented kinetic analysis supports that CapP has preference for aminocaprolactam-containing capuramycins A-503083 A and A-503083 B, and the enzyme utilizes a sequential mechanism. The results establish the first example of phosphorylation as mechanism of resistance to nucleoside antibiotics and provide the opportunity to explore the genetic prevalence of such a mechanism in Nature. Furthermore, the results provide valuable insight on the structure-activity relationship for capuramycins, which will ultimately be essential for the development of effective antibiotics for clinical use.

**EXPERIMENTAL PROCEDURES**

*Chemicals and Instrumentation*—Nucleosides and nucleotides were purchased from Sigma-Aldrich (St. Louis, MO, USA). Undecaprenyl phosphate was purchased from Larodan Fine Chemicals (Malmo, Sweden). A-503083 A, B, and F were isolated as described (9). Synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). HPLC was performed with a Waters Alliance 2695 separation module (Milford, MA, USA). HPLC was performed with a Waters Alliance 2695 separation module (Milford, MA, USA). NMR data was collected using a V arian Unity Inova 500 MHz Spectrometer (Varian, Inc., Palo Alto, CA, USA). Electrospray ionization-MS was performed in a 96-well plate in a total volume of 100 µL containing 100 mM Tris-HCl (pH 7.5), 50 mM KCl, 25 mM MgCl₂, 0.8 % Triton X-100, 166 mM undecaprenyl phosphate, 70 mM UDP-MurNAc-L-Ala-γ-D-Glu-m-DAP-[Nε-dansyl]-d-Ala-d-Ala and variable A-503083 B or 3′-phospho-A-503083 B. The reaction was initiated by the addition of MraY (0.625–2.5 mg protein), and the enzyme activity was monitored by measuring the increase in fluorescence at 535 nm (excitation at 355 nm) using EnVision 2102 Multilabel Reader (PerkinElmer, Waltham, MA, USA). The IC₅₀ was calculated using KaleidaGraph 3.6 (Synergy Software, Reading, PA, USA).

*Cloning and expression of capP*—The capP heterologously expressed in *E. coli ΔtolC* (18) was amplified using PrimeSTAR® Max DNA polymerase (Takara Bio Inc., Shiga, Japan) with the following primer pair: (forward) 5′-GCGAAGCCTTGGTCGCGACAGCACTATAG-3′/(reverse) 5′-GCGGAATTCTAGGTAGTCCGG-3′. The PCR program included an initial hold at 98 °C for 10 s, followed by 25 cycles of 98 °C for 10 s, 60 °C for 5 s, and 72 °C for 10 s. The gel-purified PCR product was cloned using pSC-B in a StrataClone Cloning Kit (Stratagene, Cedar Creek, CA, USA) to yield strata-ec-capP. After PCR fidelity confirmation, the HindIII-EcoRI fragment from strata-ec-orf21 was introduced into pUC19 (Takara Bio Inc.) at HindIII-EcoRI sites to yield pUC19-capP. The resistance to A-500359 B was tested using *E. coli ΔtolC* harboring pUC19-capP. The capP heterologously expressed in Streptomyces was also PCR-amplified using the following primer pair: (forward) 5′-GCCCATATGGATCCTTGCGCGACAGCCTATAG-3′/(reverse) 5′-GCCGCTCAGAAGCCCTTACGGATGAGTCGCG-3′. After the gel-purified PCR product was cloned into pSC-B, the resulting strata-st-capP was applied to PCR fidelity confirmation. The BamHI-HindIII
The gene for capP was amplified by PCR using Expand Long Template PCR System from Roche (Indianapolis, IN, USA) with supplied Buffer 2, 200 mM dNTPs, 5% DMSO, 10 ng pN1 (20), 5 U DNA polymerase, and 10 mM each of the following primer pair: (forward) 5'-GGTATTGAGGGTCGCACACCGA-3' (reverse) 5'-AGAGGAGATGAGGCCTACGGAGTGATGC GCGGTG-3'. The PCR program included an initial hold at 94 °C for 2 min, followed by 30 cycles of 94 °C for 10 s, 56 °C for 15 s, and 68 °C for 60 s. The gel-purified PCR product was inserted into pET30a/LIC using ligation-independent cloning as described by Novagen (Madison, WI, USA) to yield pET30-capP, which was subsequently sequenced to confirm PCR fidelity.

Assay for the CapP Reaction—The standard reaction consisted of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM ATP, 1 mM A-503083 A, B, or F, and 0.03-0.3 mg/ml CapP. The reaction was incubated at 30 °C for 30 min, and the reaction was terminated by the addition of trichloroacetic acid (7% w/v). After removal of the precipitated protein by centrifugation, the CapP-catalyzed reaction was monitored using a C-18 reversed-phase HPLC. A series of linear gradients was developed from 0.1% TFA in 2.5% acetonitrile (A) to 90% acetonitrile (B) in the following manner (beginning time and ending time with linear increase to % B): 0-8 min, 100% B; 8-18 min, 60% B; 18-25 min, 95% B; 25-32 min, 95% B; and 32-35 min, 0% B. The flow rate was kept constant at 1.0 ml/min, and elution was monitored at 260 nm. High resolution ESI mass spectra were recorded on an LTQ-Orbitrap XL (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an Agilent 1200 series Quaternary LC system (Agilent Technologies, Inc.) and Unison UK-C₁₈ (75 mm x 4.6 mm, Imtakt Co., Ltd, Kyoto, Japan). The chromatography was performed with 2.5% acetonitrile in water containing 0.1% formic acid and 10mM ammonium formate for 10 min, then with a linear gradient from 2.5 to 46.3% acetonitrile. The flow rate was kept constant at 1.0 ml/min, and elution was monitored at 260 nm.

Kinetic Analysis of CapP—Steady-state kinetic constants for CapP were determined in reactions consisting of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM ATP, variable concentrations of A-503083 A (3-1000 µM), B (3-1000 µM), or F (5-1000 µM), and CapP (10 nM, 10 nM, and 30 nM, respectively) under initial velocity conditions. To determine the kinetic constants for ATP, the conditions were identical as above except the reaction contained 0.8 mM A-503083 B, variable ATP (3-1000 µM), and 10 nM CapP. Reactions were carried out minimally in duplicate and are reported within 15% standard error.

RESULTS

Bioinformatics Analysis of CapP—BLAST analysis of CapP revealed high sequence similarity to several proteins—primarily from actinomycetes and gram-positive organisms—annotated as putative APHs. Overall CapP has the highest sequence homology to ORF21 within A-500359 biosynthetic gene cluster (72% identity/79% similarity), the gene for which has already been demonstrated to confer selective resistance to A-500359 B (18). The highest sequence homology to functionally characterized enzymes include aminoglycoside 2’-phosphotransferase from Enterococcus casseliflavus (18/30; accession no. AAC14693) that confers resistance to gentamicin (21) and aminoglycoside 3’-phosphotransferase from E. coli (18/29; accession no. P00551) that confers resistance to kanamycin and tobramycin (22). This level of sequence similarity is also observed for CapP with functionally characterized APHs that reside within the biosynthetic gene cluster for a particular aminoglycoside, including the APH from Streptomyces flavopersicus that confers resistance to spectinomycin (18/30; accession no. U70376) (23).

Heterologous Expression of capP and Capuramycin Resistance—The gene for capP along with the ermE* promoter was inserted into pWHM3 to yield pWHM3-Ep-capP. The plasmid was introduced into Streptomyces albus J1074, which is sensitive to A-500359 B, and positive transformants were incubated on ISP-2 agar media containing varying amounts of A-500359 B. In contrast to S. albus J1074 containing the empty vector, S. albus J1074 expressing capP was resistant to A-500359 B at concentrations ranging...
from 100 to 1000 µg/ml (Fig. 2A). Similarly, the gene for capP was inserted into the E. coli expression vector pUC19, and transformation into E. coli ΔtolC followed by IPTG induction yielded the strain E. coli ΔtolC/pUC19-capP that was—in contrast to controls—resistant to A-503083 B at concentrations ranging from 10 to 1000 µg/ml (Fig. 2B).

Functional Assignment of CapP as a Capuramycin 3’′-Phosphotransferase—The gene for capP was cloned into pET30Xα/LIC for expression in E. coli BL21(DE3). The recombinant protein was soluble and purified using immobilized metal affinity chromatography. SDS-PAGE analysis revealed a dominant band with the expected size for His$_6$-CapP (Fig. 3A). When CapP was incubated with A-503083 B and ATP using typical assay conditions for APHs, a new peak appeared with the simultaneous decrease of the peak corresponding to A-503083 B (Fig. 3B). In addition, the peak for ATP decreased with the concomitant increase of a new peak that co-eluted with authentic ADP. The unidentified peak was collected and analyzed by LC-MS to yield an (M + H)$^+$ ion at $m/z = 693.1$, consistent with the molecular formula of C$_{24}$H$_{32}$O$_{16}$N$_8$P corresponding to mono-phosphorylated A-503083 B [expected (M + H)$^+$ ion at $m/z = 693.1$]. The product was further analyzed using high resolution LC-MS to yield an (M-H)$^-$ ion at $m/z = 691.16040$, consistent with the molecular formula of C$_{24}$H$_{32}$O$_{16}$N$_8$P corresponding to mono-phosphorylated A-503083 B [expected (M - H)$^-$/LI_1 ion at $m/z = 691.16069$] (Supplemental Fig. S1).

To confirm the MS results and assign the regiochemistry of phosphorylation, the CapP product was analyzed using NMR spectroscopy. The $^1$H-NMR spectrum of the substrate A-503083 B was acquired in D$_2$O and the chemical shifts assigned in part with the aid of the reported assignments (9). The $^1$H-NMR spectrum of the CapP product revealed a downfield shift of the 2” proton from 4.20 ppm to 4.38 ppm and a downfield shift of the 3” proton from 4.57 ppm to 5.05 ppm (Supplemental Fig. S2), which was confirmed by analysis of the $^1$H COSY spectrum (Supplemental Fig. S3). The $^{13}$C-NMR spectrum of the substrate A-503083 B and the CapP product were indistinguishable excluding only the downfield shift of C-3” from 61.75 ppm to 66.10 ppm (Supplemental Fig. S4). Thus, the data are consistent with the identity of the CapP product as 3’′-phospho-A-503083 B.

Bioactivity of the CapP Product—The CapP product, 3’′-phospho-A-503083 B, was tested for antimicrobial activity against Mycobacterium smegmatis SANK 70186. In contrast to control experiments using A-503083 B, no zone of inhibition was observed using two different concentrations of 3’′-phospho-A-503083 B (Fig. 4A). The CapP product was also tested for the ability to inhibit E. coli MraY activity in vitro using a previously developed fluorescence-based assay (7). Activity of MraY with varying amounts of A-503083 B (0.0016 – 25 µg/ml) yielded an IC$_{50} = 0.0046$ µg/ml (~8 nM) (Fig. 4B). Activity of MraY with varying amounts of 3’′-phospho-A-503083 B (0.0016 – 25 µg/ml) yielded an IC$_{50} = 1.25$ µg/ml (~1.8 µM). Thus, a 270-fold increase in IC$_{50}$ is observed following phosphorylation of A-503083 B by CapP.

Substrate Specificity and Single-Substrate Kinetics—The activity of CapP was tested with alternative substrates, A-503083 A and A-503083 F. HPLC analysis revealed the appearance of a new peak in both cases with the simultaneous decrease in the peak corresponding to substrate (Fig. 3B and D). The unidentified peak was collected and analyzed by LC-MS to yield an (M + H)$^+$ ion at $m/z = 707.1$, consistent with the molecular formula for the mono-phosphorylated A-503083 A [expected (M + H)$^+$ ion at $m/z = 707.2$] and an (M - H)$^-$/LI_1 ion at $m/z = 581.1$, consistent with the molecular formula for the mono-phosphorylated A-503083 F [expected (M - H)$^-$/LI_1 ion at $m/z = 581.1$].

Single-substrate kinetic experiments were performed with A-503083 A, B, and F using near-saturating ATP (1 mM). Initial velocity plots using variable A-503083 A or A-503083 B revealed Michaelis-Menten kinetics yielding a $K_m = 175 \pm 30$ µM and $k_{cat} = 46 \pm 3$ min$^{-1}$ for A-503083 A and $K_m = 170 \pm 40$ µM and $k_{cat} = 34 \pm 4$ min$^{-1}$ for A-503083 B (Fig. 5A and B). Initial velocity plots using variable ATP and near-saturating A-503083 B revealed Michaelis-Menten kinetics yielding a $K_m = 180 \pm 75$ µM and $k_{cat} = 41 \pm 6$ min$^{-1}$ (Fig 5C). Initial velocity plots using variable A-503083 F with saturating ATP did not follow typical Michaelis-Menten kinetics; however, the data are consistent with an apparent maximal turnover of ~0.2 min$^{-1}$ (Supplemental Fig. S5).
**Bi-substrate Kinetic Analysis**—The bi-substrate initial velocity pattern for CapP was analyzed using varied ATP and A-503083 B. Reciprocal plots revealed the lines intersected to the left of the vertical axis consistent with a sequential kinetic mechanism (Fig. 5D). Kinetic constants derived by fitting the data to a bi-substrate sequential mechanism (24) were $K_{m}$ = 180 µM for A-503083 B and $K_{m}$ = 200 µM for ATP, similar to the values calculated using single-substrate kinetic analysis.

**DISCUSSION**

Capuramycins effectively and rapidly kill several species of mycobacteria in vitro (25, 26), and they have recently been demonstrated to be very effective and non-toxic anti-TB drugs using a mouse model (25, 27), suggesting these compounds have clinical promise. Given the exciting potential of this family of antibiotics, it is of significant interest to elucidate the potential mechanism(s) of resistance to capuramycins prior to utilization in the clinic, thus serving as the foundation for limiting the onset of capuramycin-resistant strains that is an inevitable consequence of the selection process.

We had previously demonstrated that heterologous expression of orf21, encoding the CapP homolog encoded within the A-500359s biosynthetic gene cluster, was sufficient to confer a high-level of resistance to A-500359 B using model gram-positive and gram-negative strains, but the molecular details of this resistance were only inferred from sequence analysis (18). Therefore, we set out to establish the biochemical function of CapP. As expected, CapP utilizes ATP to generate the mono-phosphorylated product, and consistent with the heterologous expression experiments, the phosphorylated product does not exhibit antibacterial activity against a model mycobacterial strain, *M. smegmatis* SANK 70186. Furthermore, this resistance is manifested in part by a significant loss in the ability to inhibit the MraY-catalyzed reaction and not solely due to other factors such as a decrease uptake into the cytoplasm.

Phosphorylation as a mechanism of resistance has previously been demonstrated for the structurally-unrelated, adenine-derived nucleoside antibiotic A201A from *Streptomyces capreolus* (28). In this case, using cell-free extracts of *S. lividans* expressing ard2, the gene product was shown to phosphorylate the 2-hydroxyl of the hexa-furanose component of A201A, and the phosphorylated product lost its antibacterial activity against *Micrococcus luteus* (28). Subsequently, it was determined that the ard2 gene is likely located within the biosynthetic gene cluster for A201A (29). Therefore, the combined results from studies with CapP and Ard2 suggest that phosphorylation by a gene product encoded within the biosynthetic gene cluster may be a common strategy for self-resistance to nucleoside antibiotics, which also includes other previously identified strategies such as acetylation (30). This finding also suggests that the 3'-hydroxyl of capuramycins is important for activity and provides new insights into the structure-activity relationship for these compounds.

While mass analysis confirmed a single phosphate was incorporated into the CapP product, the regio-specificity of phosphorylation was only revealed using NMR spectroscopic analysis. The 3'-$H$ in the 1H NMR spectrum of A-503083 B shows a clear downfield shift from 4.57 ppm to 5.05 ppm in the CapP product. The magnitude of this change (0.48 ppm) is comparable to that observed for the 3'-$H$ of capuramycins. This is perhaps not surprising given the preference for aminocaprolactam-containing capuramycins. This is perhaps not surprising given that A-503083 A (IC$_{50}$ = 0.024 µM) and A-503083 B (IC$_{50}$ = 0.038 µM) are much better inhibitors of MraY than A-503083 F (IC$_{50}$ = 17.9 µM) (9). It is therefore imperative that, when A-503083 A is produced from A-503083 F as we have demonstrated elsewhere (20), the organism is able
to protect itself. Analysis of the second-order rate constants suggest a slight preference for A-503083 A (4.5 x 10^3 M^-1 s^-1) compared to A-503083 B (3.3 x 10^3 M^-1 s^-1), which is also remarkably consistent with the observed inhibition activity of these compounds. The magnitude of these second order rate constants is slightly less to that observed for most APHs (typically near 10^5 M^-1 s^-1) (19, 32).

Most APHs display substrate inhibition patterns with respect to the aminoglycoside substrate, and analysis of the kinetic plots for A-503083 A and B suggest this may also be the case at > 1 mM substrate. Kinetic analysis with variable ATP revealed that, at relatively low ATP concentration, the velocity appears greater than expected. This suggested that perhaps the enzyme co-purifies with ATP, however no reaction was observed without adding ATP; further kinetic analysis is required to address this observation. Bi-substrate kinetic analysis of CapP was consistent with a sequential kinetic mechanism, which is also observed for most APHs and provides further evidence for the relationship of CapP and APHs (32). Therefore the properties of CapP appear similar to most APHs, yet examination of the structures of aminoglycosides and capuramycins reveal significant differences, and the structural and mechanistic details of CapP that yield such a dramatic variation in substrate specificity are currently being investigated.

Microorganisms utilize a variety of resistance mechanisms to protect themselves from endogenous and exogenous antibiotics, such as specialized transport and binding proteins, chemical modification of the drug target, and modification of the antibiotic itself (33). We have now demonstrated the last strategy—modification of the antibiotic—is observed for self-resistance to capuramycins that is manifested by the activity of CapP. Likewise, aminoglycosides are rendered inactive by APHs, and to date several APHs have been identified within pathogenic bacterial strains such as *E. coli* (34) and *S. aureus* (35) as well as aminoglycoside-producing strains (36), for example, the neomycin (37), ribostamycin (38), and streptomycin (39) producing strains, that often contain a gene for an APH within the biosynthetic gene cluster that is utilized for self-resistance (37-39). Interestingly, despite overall low sequence identity, the APHs from aminoglycoside-producing and non-producing strains appear to have an evolutionary relationship (40). With the function of CapP now assigned, it is possible to search the enormous amount of data generated from whole-genome sequencing to perform a similar analysis and to identify potential candidates in other organisms that may have the same function as CapP. The details of these studies, along with the data presented here, will be critical for the successful implementation of capuramycins as antibiotics.

REFERENCES

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The abbreviations used are: APH, aminoglycoside phosphotransferase; MDR, multiple drug resistance;

FIGURE LEGENDS

FIGURE 1. Structures of representative capuramycins that were discovered using a screen to isolate specific inhibitors of MraY.

FIGURE 2. Heterologous expression of capP conferring resistance to A-500359 B. A, S. albus J1074 with pWHM3-Ep (control) or pWHM3-Ep-capP grown on ISP2 media containing different concentrations of antibiotic. 1/100 and 1/1000 indicate dilution ratios of cultured mycelium spotted on the agar media. B, E. coli ΔtolC with pUC19 (control) or pUC19-capP grown on LB media containing different concentrations of antibiotic and 1 mM IPTG for induction of expression.

FIGURE 3. In Vitro Characterization of CapP. A, SDS-PAGE of purified CapP (expected MW of 37.8 kD). Lane 1, MW markers and lane 2, purified His$_6$-CapP. B-D, HPLC analysis of the activity of CapP showing the time-dependent formation of a new peak with a mass consistent with the mono-phosphorylated product and the simultaneous loss of the substrate peak.


FIGURE 5. Kinetic Analysis of CapP-catalyzed Reaction. A, Single substrate kinetic analysis using near saturating ATP and variable A-503083 A. B, Single substrate kinetic analysis using near saturating ATP and variable A-503083 B. C, Single substrate kinetic analysis using near saturating A-503083 B and variable ATP. D, Bi-substrate kinetic analysis using variable ATP and multiple fixed concentration of A-503083 B at 1 µM (●), 10 µM (▲), 50 µM (▼), and 200 mM µM (○).
Fig. 1

A-500359 A \( (R^1 = \text{CH}_3, R^2 = \text{H}) \)
A-500359 B \( (R^1 = R^2 = \text{H}) \)
A-503083 A \( (R^1 = \text{CH}_3, R^2 = \text{CONH}_2) \)
A-503083 B \( (R^1 = \text{H}, R^2 = \text{CONH}_2) \)
Fig. 2

A

A-500359 B (μg/mL)

0 100 200 500 1000

1/100

S. albus / pWHM3-Ep

1/1000

S. albus / pWHM3-Ep-capP

1/100

1/1000

B

0 10 100 1000

E. coli ΔtolC / pUC19

E. coli ΔtolC / pUC19-capP
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