Mutations M219K, S266A, and G337S in transcription termination factor Rho have been shown to confer resistance to the antibiotic bicyclomycin (BCM). All three His-tagged mutant Rho proteins exhibited similar $K_m$ values for ATP; however, the $V_{max}$ values at infinite ATP concentrations were one-fourth to one-third that for the His-tagged wild-type enzyme. BCM inhibition kinetics of poly(C)-dependent ATPase activity for the mutant proteins were non-competitive with respect to ATP (altering catalytic function but not ATP binding) and showed increased $K_i$ values compared with His-tagged wild-type Rho. M219K and G337S exhibited increased ratios of poly(U)/poly(C)-stimulated ATPase activity and lower apparent $K_m$ values for ribo(C)$_{10}$ in the poly(dC)-ribo(C)$_{10}$-dependent ATPase assay compared with His-tagged wild-type Rho. The S266A mutation did not show an increased poly(U)/poly(C) ATPase activity ratio and maintained approximately the same $K_m$ for ribo(C)$_{10}$ in the poly(dC)-ribo(C)$_{10}$-dependent ATPase assay. The kinetic studies indicated that M219K and G337S altered the secondary RNA binding domain in Rho whereas the S266A mutation did not. Transcription termination assays for each mutant showed different patterns of Rho-terminated transcripts. Tyrosine substitution of Ser-266 led to BCM sensitivity intimating that an OH (hydroxyl) moiety at this position is needed for BCM (binding) inhibition. Our results suggest BCM binds to Rho at a site distinct from both the ATP and the primary RNA binding domains but close to the secondary RNA-binding (tracking) site and the ATP hydrolysis pocket.

Bicyclomycin (BCM)\(^1\) is a structurally novel antibiotic biosynthetically derived from leucine and isoleucine (1, 2). First isolated in 1972 from Streptomyces sapporosensis (3–5) and Streptomyces aizumenses (6, 7), BCM has been shown to possess antimicrobial activity against Gram-negative bacteria such as Escherichia coli, Shigella, and Salmonella (8) and has been introduced under the trade name of Bicozamycin for the treatment of nonspecific diarrhea in calves and swine. Recently, BCM (Structure 1) was introduced under the trade name of Bicozamycin for the treatment of nonspecific diarrhea in humans and bacterial infection 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.

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\‡ The abbreviations used are: BCM, bicyclomycin; DTT, dithiothreitol; PCR, polymerase chain reaction; RF DNA, replicative form of M13 DNA.

BCM targets Rho transcription termination factor, an essential protein in Escherichia coli (10). BCM resistance was achieved using UV irradiation to produce cells with Rho mutations. Sequence analysis of 20 mutants showed that a single base change occurred in the $rug$ gene at one of three different locations, M219K, S266A, and G337S (10). Isolation and assay of a crude preparation of the G337S Rho protein showed that at a constant ATP concentration, the poly(C)-dependent ATPase activity was insensitive to BCM, but the wild-type protein was sensitive (10). Subsequent studies on the BCM-Rho inhibition process have not identified the BCM-binding site.

Transcription termination in Enterobacteriaceae is a regulatory process that controls gene expression (11, 12). Transcription termination proceeds by one of two major mechanisms. In one process, nascent RNA is released at intrinsic termination sites along the E. coli genome. Termination (Rho-independent) generally proceeds after an upstream portion of the DNA–RNA hybrid of the transcription bubble is destabilized by competitive formation of an RNA GC-rich hairpin followed by a poly(rU) sequence that disrupts the transcription process. In the second mechanism, Rho factor terminates transcription. Rho first binds to specific cytidine-rich areas on the newly synthesized RNA (the rut site) and then tracks toward the 3′ end of the RNA where the stalled RNA polymerase rests on the transcription bubble (13). The movement of Rho toward the RNA polymerase is fueled by Rho-dependent ATPase activity (14). In a poorly understood mechanism that may involve Rho helicase activity (15), Rho factor disrupts the polymerase complex and terminates transcription (16–18).

Active Rho is a hexamer of identical 419-amino acid residue proteins (19) with binding domains for the rut RNA site (the primary RNA binding domain) and ATP (20). The tertiary structure of Rho factor is only partially defined (21, 22). The primary RNA-binding site is localized in the N terminus of Rho (23, 24), and this binding can be measured by a filter binding assay using radiolabeled poly(C) (25). Photochemical irradiation of the overexpressed 1–116-amino acid fragment and ribo(C)$_{10}$ gave a cross-linked adduct and defined the minimum protein sequence required for RNA binding (23). Sequence comparison of this fragment identified the RNA recognition motif, GGFF, an RNA binding domain conserved motif, located between residues 61 and 64 (26, 27). Subsequent site-directed mutational analysis of the phenylalanine residues led to decreased binding for the RNA trp $t′$ fragment that contains a Rho-dependent termination site. An NMR structure of the N-terminal 130-amino acid fragment has been recently determined (21), and the crystal structure of this fragment has been solved (22).

The entire Rho protein has resisted crystallization. However, comparison of the amino acid sequences of Rho and F$_1$-ATP synthase, another ATP-binding protein, yielded 21% sequence identity and 43% similarity in a pairwise comparison (28), permitting a working model of Rho to be built (29). Although

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Identification of the Bicyclomycin Binding Domain through Biochemical Analysis of Antibiotic-resistant Rho Proteins*

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Identifying the Bicyclomycin Binding Domain

The amino acid sequence of the first 130 amino acid residues did not align well with F$_1$-ATP synthase, they do share a common N-terminal $\beta$-barrel, which in Rho is similar to an oligosaccharide/oligonucleotide-binding fold (22). This observation strengthens the use of F$_1$-ATP synthase as a suitable structural model for Rho. The ATP binding/hydrolysis domain in Rho is located after amino acid 167 in the C-terminal half of Rho (24, 30–31) and is similar in primary structure to F$_1$-ATP synthase. Rho ATPase activity can be monitored by a polyribo-nucleotide-dependent ATPase assay (14). In vitro experiments showed that poly(C) stimulated the latent ATPase activity of Rho by binding to both a primary RNA-binding site and to a secondary (tracking) RNA site (31). A secondary RNA-binding site was proposed after strongly binding poly(dC) failed to stimulate ATPase activity (32, 33). Adding short oligoribonucleotides (at concentrations that by themselves do not stimulate ATPase activity) in the presence of poly(dC) initiated ATP hydrolysis at rates comparable to those observed with poly(C) alone. This finding indicated that poly(dC) binds to the primary RNA-binding site but not to the secondary site. The secondary RNA-binding site appears distinct from the primary binding domain, and the Rho–RNA interactions are weaker permitting the movement of Rho toward the RNA polymerase (16, 34). Mutations on Rho that altered the kinetics of ribo(C)$_{10}$-stimulated Rho ATPase activity in the presence of poly(dC) suggest that the secondary RNA-binding site was altered (33, 35). The secondary site mutations mapped to the F$_1$-ATP synthase-based model did not provide a well-characterized binding domain (29).

Kinetic analysis of the BCM–Rho interaction showed that BCM inhibits poly(C)-stimulated ATPase activity by a reversible, noncompetitive pathway with respect to ATP (36). In an in vitro transcription termination assay, BCM inhibited production of Rho-dependent transcripts (37). At BCM concentrations that yielded 50% inhibition, transcripts were observed midway to the stalled polymerase (37). We found that BCM exhibited mixed type inhibition kinetics in the poly(dC)/ribo(C)$_{10}$-stimulated ATPase assay which showed that the antibiotic altered binding of ribo(C)$_{10}$ to Rho, and the $K_i$ for BCM was altered by ribo(C)$_{10}$. This result suggested that BCM inhibited RNA binding at the secondary site in Rho, leading to the loss of the ATPase activity that is necessary for Rho translocation to the RNA polymerase (18, 37).

The BCM receptor site on Rho has not been identified. By comparison, much is known about the structural requirements in BCM for Rho binding. A structure-activity relationship study has determined that the C(1) triol and the [4.2.2]-piperazine ring system are necessary for drug binding but the C(5)/C(5a) exomethylene group is not (38–42). Recently, an aryl aldehyde derivative of BCM was shown to inhibit transcription termination in a manner similar to BCM and led to permanent inactivation of Rho when NaBH$_4$ was added (43, 44). The site of addition was found to be Lys-181 (44), providing preliminary information on BCM binding. Significantly, Platt and co-workers (31) have shown that Lys-181 is also near the catalytic ATP hydrolysis site. A complementary approach to elucidate the BCM binding pocket in Rho is to analyze the effect of site mutations in Rho that alter drug binding and the kinetic properties of the protein.

In this work, the kinetic behavior of three BCM-resistant mutant Rho proteins, M219K, S266A, and G337S, and a site-directed mutant S266Y are characterized. The location of these mutations on a putative model of Rho based on the F$_1$-ATP synthase structure for bovine mitochondrial enzyme is discussed in context of their biochemical behavior.

**Experimental Procedures**

BCM was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), and was further purified by three successive silica gel chromatographies using 20% methanol/chloroform as the eluant (36). Bacterial phase M13 mp19RF DNA and polynucleotides were purchased from Amersham Biosciences Biotech. Oligonucleotide primers were synthesized by Genosys Biotechnological, Inc. (The Woodlands, TX). *E. coli* strain SDM, T7 DNA polymerase, and 5-methyl-dCTP were purchased from U. S. Biochemical Co., and T4 polynucleotide kinase, T4 DNA ligase, and restriction enzymes were from Promega Co. (Madison, WI). pLex DNA polymerase was obtained from Stratagene (La Jolla, CA). Talon metal affinity resin was from CLONTECH Laboratories (Palo Alto, CA). [$\gamma$-32P]ATP and [$\alpha$-32P]UTP (3000 Ci/mmol) were purchased from NEN Life Science Products, and nucleotides and RNase inhibitor were from Ambion, Inc. (Austin, TX). Polyethyleneimine thin layer chromatography plates used for ATPase assays were purchased from J. T. Baker, Inc. Ribo(C)$_{10}$ was obtained from Oligos Etc. (Wilsonville, OR). All other chemicals were reagent grade.

**Overexpression Vectors—Rho DNA (wild-type)** was amplified from the p39-ASE plasmid using PCR technology with *Pfu* polymerase. Mismatched primers 5'-AGAATCTACACCATATGAACTTTCAGC-3' and 5'-CAGGAATTTCTCTCATATGGCCGCACCGC-3' creating unique *Nde* I sites at the start of translation and downstream from the gene at base position 1527 were used to amplify the *rho* gene. The resulting DNA was digested with *Nde* I and ligated into the *Nde*I-linearized pET-11b expression vector. Transformants introduced into JM107 host cells were identified for proper orientation and sequenced to ensure no unwanted substitutions were introduced from the amplification step. The plasmid PET-RhoW was introduced into BL21(DE3)pLysS, and the expression of Rho protein was induced with addition of 1 mM isopropyl-$\beta$-D-thiogalactopyranoside at a cell concentration of 0.8 A$_{600}$ of 0.6. After 4 h incubation at 37 °C, cells were harvested by centrifugation and suspended in 10 mM Tris-HCl, 100 mM NaCl, 10% glycerol, 1 mM $\beta$-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0 buffer (buffer A). The cells were treated with lysozyme at 0.5 mg/ml for 20 min at 0 °C and then 4 mM DTT was added. The lysed cells were placed on ice for 10 min after which they were subjected to brief sonication disruption. The broken cells were centrifuged, and the soluble proteins were loaded on a Talon metal affinity resin. The column was washed with buffer A alone, buffer A with 10 mM imidazole, and buffer A with 50 mM imidazole, and the protein was eluted with buffer A and 200 mM imidazole. Peak fractions were collected and dialyzed overnight against 10 mM Tris-HCl, 100 mM NaCl, 50% glycerol, 1 mM DTT, pH 8.0, and stored at −20 °C. The isolated protein showed no visible contamination with other proteins on SDS-polyacrylamide gel electrophoresis.

**Construction of Mutant Overexpression Vectors—The rho gene from** BCM-resistant *E. coli* strains BCM102, BCM108, and BCM110 (10) were amplified with primers flanking the *Bcl* I and *Kpn* I restriction sites. The resulting amplified DNA was digested with *Bcl* I and *Kpn* I and ligated into the *Bcl* I-*Kpn* I linearized PET-RhoW plasmid. The PET-RhoW plasmid was grown in the dam strain, SCS110, to allow the digestion of the *Bcl* I site. Recombinant plasmids were transformed into the host strain JM107. Plasmids showing correct restriction patterns were isolated. Plasmids were transformed into BL21(DE3)pLysS, and mutant Rho proteins were overexpressed as described for the wild-type Rho.

**Subcloning of rho Gene into Phage M13mp19 Vector—**By using PCR amplification of plasmid DNA containing the wild-type *rho* gene with two synthetic oligonucleotide primers, a SalI restriction site was introduced. The PCR fragment was digested with HindIII and KpnI and ligated into the HindIII-KpnI linearized pET-RhoW plasmid. The PET-RhoW plasmid was grown in the dam strain, SCS110, to allow the digestion of the SalI site.
other primer, d(GTGGAGGCGTCTGTCGCGA), was complementary to the wild-type rho gene at nucleotide positions 1575–1594. The PCR product was digested with Ssfl and KpnI, purified from agarose, and ligated into the Ssfl- and KpnI-linearized bacterial plasmid M13mp19 RF DNA. Single-stranded phage DNA was isolated from cells and sequenced to confirm the sub cloning of wild-type rho gene.

**Polymerase Chain Reactions**—PCR reactions were carried out in buffer solution containing 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.2 mM dNTPs, 0.4 μM of each oligonucleotide primer, 4 mM MgCl₂, and 0.1 μg of plasmid DNA in 0.05 ml total volume at 95 °C for 10 min followed by the addition of T4 DNA polymerase.

**Oligonucleotide Site-directed Mutagenesis**—Single-strand M13 phage DNA containing the wild-type rho gene was isolated from E. coli strain SDM by infection with the phage particles obtained from E. coli strain JM109. Oligonucleotide primer 5'-CGAGTTGACTGCGAAG-3' bearing the Tyr codon was phosphorylated using T4 polynucleotide kinase. The second strand (mutant strand) was selectively methylated by incorporating 5-methyl-dCTP during the synthesis reaction using T7 DNA polymerase. The resulting hemi-methylated DNA was digested with MspI followed by treatment with Klenow. Un Copy single-strand DNA was removed by adding HhaI during restriction digestion. The remaining mutated methylated single-strand DNA was then transformed into E. coli strain SDM. Mutants were selected by isolating single-strand phage DNA and identified by DNA sequencing using M13 (~40) universal primer. Phage that contained the desired mutated rho gene were allowed to infect the E. coli strain SCS110. The mutated rho genes were then removed from phage RF DNA by treatment with BclI and KpnI, and the resulting fragment was ligated into the BclI and KpnI sites of the linearized His-tagged Rho overexpression vector pET-RhoW. The plasmids were transformed into E. coli strain BL21(DE3)pLysS.

**In Vitro Termination Assays**—Transcription termination assays were carried out using a modified trp operon (45) as described (37). Briefly, the reaction was carried out in a 10-μl volume with 200 μM ATP, CTP, GTP, and 20 μM UTP, 7 μCi of [α-32P]UTP, 0.1 pmol of nucleotide primer, 0.4 units/ml RNase inhibitor, 0.01 μg/ml E. coli RNA polymerase, and 70 μM Rho protein or absence of BCM. The reactions were carried out for 20 min at 37 °C, diluted with 100 μl of 0.5 M sodium acetate, 1 mM EDTA, and carrier RNA to 0.8 mg/ml, extracted with phenol, precipitated with ethanol, and dissolved in gel loading buffer. The RNA transcripts were separated on a 5% polyacrylamide gel containing 8 μM urea and were visualized by drying the polyacrylamide gel electrophoresis gels and allowing them to expose a PhosphorImager plate (Fuji Photo Film Co., Japan) for approximately 30 min. Exposed plates were scanned using a Fuji BAS1000 BioImaging Analyser. The data were collected and analyzed using a MacNtosh BAS analysis program. Quantitation of the termination assays was done by subtracting the digitized background from both the read-through transcript and the Rho-terminated transcripts. Percent of termination by Rho was determined as the amount of Rho-terminated transcripts divided by the read-through transcript plus the Rho-terminated transcripts.

**ATPase Activities**—The poly(C)-dependent ATPase activity was measured as described (36). Briefly, reactions were initiated by adding ATP (9.1–100 μM) and 0.5 μCi of [32P]ATP to the solution containing 40 mM Tris-HCl, pH 7.9, 50 mM KCl, 12 mM MgCl₂, 0.1 mM EDTA, 0.1 mM DTT, 0.07 mg of bovine serum albumin, 24 μM poly(C), and 70 mM Rho protein or mutants. Aliquots were removed at various times after the start of the reaction, and the 32P-labeled inorganic phosphate and ATP were separated on Baker-Flex cellulose polyethyleneimine TLC plates using 0.75 M potassium phosphate, pH 3.5, as the mobile phase (46). The TLC plates were exposed to PhosphorImager plates for 15-30 min and scanned using a Fuji BAS1000 BioImaging Analyzer and analyzed using a MacNtosh BAS analysis program. The initial rates of the reactions were determined by plotting the amounts of ATP hydrolyzed versus time.

**Poly(C)-dependent ATPase Activities**—The poly(C)-dependent ATPase activities were carried out in 20-μl reactions containing 30 mM Tris acetate, pH 7.9, 0.4 mM magnesium acetate, 10 mM DTT, 0.2 mM ATP, 0.015 μCi of [γ-32P]ATP, 3.2 μM poly(dC)21 ribo(C)10 (0.7–14 μM), 18 nm Rho protein, and 0–1000 μM BCM. Reactions were preincubated at 32 °C for 2 min prior to adding ATP. Aliquots (2 μl) were removed periodically (15, 30, 45, 60, and 90 s) during the reaction and spotted onto the TLC plates. Plates were analyzed as described previously.

**DNA Binding Assays—**Polyctydylate (poly(C)) was labeled at the 5' end using [γ-32P]ATP in reactions containing 50 pmol of poly(C), 70 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 30 μCi of [γ-32P]ATP (3000 Ci/mmol), and 10 units of T4 polynucleotide kinase. The reaction mixture was incubated at 37 °C (30 min) and then at 70 °C (10 min) and loaded on a polyacrylamide gel. The desired length of oligomer (approximately 150 nucleotides) was cut out, eluted, and recovered by ethanol precipitation. Binding of poly(C) to Rho was done in binding buffer (40 mM Tris-HCl, pH 8.0, 25 mM KCl, 10 mM MgCl₂, 0.1 mM DTT, and 0.1 mM EDTA) and 0–113 nm Rho in a total volume of 100 μl. Rho with or without BCM (1 μM) was preincubated at 4 °C (2 min), and approximately 25 pCi (final concentration) of 5' end 32P-labeled poly(C) was added. Each reaction mixture was incubated at 37 °C (1 min) followed by filtration of 50 μl of the reaction solution using 25-mm HA nitrocellulose filters presoaked with 0.1 mg/ml RNA. The filters were washed twice with 0.5 ml of buffer, dried briefly, and measured in a scintillation counter.

**RESULTS**

**Protein Overexpression and Cloning Strategies**—Our investigation focused on mutations introduced at amino acid positions 219, 266, and 337 in Rho, since single amino acid changes at these sites conferred BCM resistance. These Rho proteins were expressed with a His-tag using the pET-14b vector. The wild-type rho gene was PCR-amplified with Pfu polymerase using an upstream primer with a single base change thus creating a new NdeI site at the start codon. The amplified DNA was digested with NdeI and ligated into pET-14b at the NdeI site. Protein expression was induced by inducing the T7 polymerase (under lac control) by the addition of isopropyl-β-thiogalactopyranoside to the cells. A mutagenesis cartridge was constructed to allow overexpression of mutant Rho protein using the wild-type overexpression system. This was accomplished by replacing the BclI-KpnI DNA fragment from the wild-type plasmid with the BclI-KpnI fragment isolated from M219K, S266A, and G337S. The wild-type plasmid was grown in SCS110 (dam -), allowing cleavage with BclI. PCR-amplified rho genes from mutant Rho cells were digested with BclI and KpnI and were directionally cloned into this site. The BclI-KpnI fragment contained amino acids 14–364, which covered the range of mutations in BCM resistant Rho. The M219K, S266A, and G337S were overexpressed and isolated.

**Comparison of the Wild-type and His-tagged Wild-type Rho**—The kinetic parameters for wild-type and His-tagged wild-type Rho were compared in the poly(C)-stimulated ATPase assay (Table 1), and we observed little differences between them. The Kₘ values for ATP for both proteins were the same at 13 μM; the Vₘₐₓ for wild-type Rho was 18.1 μmol/min/mg and 14.3 μmol/min/mg for His-tagged wild-type Rho. We also compared...

### Table 1

| Rho protein | I₅₀ values for BCM | Polycl(C)-stimulated ATPase assay | Kₘ for BCM | Kₘ for ATP | Vₘₐₓ for ATP hydrolysis | Polycl(C)-ribo(C)₁₀ ATPase activity, Kₘ for BCM |
|-------------|-------------------|-----------------------------------|------------|------------|------------------------|-----------------------------------------------|
| Wild-type (wt) | 60                | 25                                | 13.3       | 15.1       | 2.0                    |                                |
| His-tagged wt | 60                | 34                                | 13.2       | 14.3       | 2.0                    |                                |
| M219K-His    | 200               | 275                               | 15.4       | 4.8        | <0.1                   |                                |
| S266A-His    | 1000              | 480                               | 14.3       | 4.4        | 4.0                    |                                |
| G337S-His    | 400               | 440                               | 11.1       | 7.7        | <0.1                   |                                |
| S266Y-His    | 60                | 25                                | 16.0       | 13.0       | 2.0                    |                                |
their RNA specificities and transcription termination properties and found no significant differences (Table II). These results showed that wild-type Rho can be overexpressed in the pET-14b vector system and that adding the His-tag unit did not alter Rho ATPase activity appreciably. This finding is consistent with previously published results using His-tagged Rho proteins (29).

**BCM Inhibitory Properties of Overexpressed Rho Proteins—** The identical BCM IC50 value (60 μM) was observed for both wild-type and His-tagged wild-type Rho in the poly(C)-stimulated ATPase assay (Fig. 1 and Table I). The BCM IC50 values for the His-tagged M219K, G337S, and S266A were 200, 400, and 1000 μM, respectively. These values showed that the mutants were 3–16 fold less sensitive to BCM than the His-tagged wild-type protein.

**Kinetics of BCM Inhibition of Poly(C)-stimulated ATPase Activity—** The BCM inhibition kinetics for the three mutant Rho proteins in the poly(C)-stimulated ATPase assay was determined by varying the ATP concentrations between 9.1 and 100 μM. BCM concentrations were varied between 0 and 1000 μM, depending on the mutant protein. Non-competitive inhibition kinetics with respect to ATP were seen for all three proteins when the data were graphed using double-reciprocal plots (data not shown). A similar finding was reported for wild-type proteins when the data were graphed using double-reciprocal plots (36). For M219K, the Km for ATP was 15 μM, which is close to the value seen for wild-type Rho (Km = 13 μM); however, the Vmax of the reaction in the absence of BCM was only 27% of the rate of wild-type Rho and 34% of His-tagged wild-type Rho. Similarly, the Km values for S266A and G337S BCM-resistant His-tagged Rho mutants were comparable to the wild-type Rho, and the Vmax values were 24 and 43% of the wild-type and 31 and 54% of the His-tagged wild-type Rho, respectively (Table I). The slopes (Km(app)/Vmax(i)) and intercepts (1/Vmax(i)) from the double-reciprocal plot were charted against BCM concentration for the M219K mutant and gave a Ki = 275 μM for BCM inhibition (Table I). Similarly, the Km values for BCM inhibition for S266A and G337S were 480 and 440 μM, respectively (Table I). The kinetic properties of Rho measured by the poly(C)-dependent ATPase activity were not altered by replacing the serine with tyrosine at position 266 and provided no BCM resistance (Table I). Interestingly, the Vmax of this mutant was nearly the same as that of the His-tagged wild-type Rho suggesting that the hydroxyl group is necessary for BCM binding to Rho and also aids in ATP hydrolysis.

**The Stimulation of ATPase Activity of the Rho Mutant Proteins with Varying RNA Substrates—** The latent ATPase activity of Rho was measured using different RNAs to stimulate the Rho mutant proteins. Poly(C)-stimulated ATPase activity was compared with the poly(U)-, poly(I)-, and poly(A)-stimulated ATPase activities. As seen in Table II, the percentage of poly(U) to poly(C) activity was near 8% for the wild-type and 1.8% for the His-tagged wild-type Rho. This increased to 30 and 44% for M219K and G337S, respectively, whereas S266A showed <0.2% of the poly(C)-stimulated activity when poly(U) was used to stimulate the activity. M219K and G337S also exhibited slightly higher percentages of ATPase activities using either poly(I) or poly(A) versus poly(C) when compared with the other Rho proteins. On the whole, these percentages were smaller than the poly(C) rates. Results for M219K and G337S suggest that RNA specificity was altered by these mutations and also led to BCM resistance. The S266A and S266Y mutants showed the same type of poly(U) activity as the wild-type Rho, suggesting that these mutations were not activated by poly(U) and maintained specificity for poly(C) at the secondary RNA-binding site (Table II).

**Mutations Alter the Km for Ribo(C)10 in the Poly(dC):Ribo(C)10-stimulated ATPase Assay—** Ribo(C)10 stimulates the latent ATPase activity of Rho when poly(dC) is bound to the primary RNA-binding site (32, 34). The ATPase activity was measured as a function of the ribo(C)10 concentration for each of the BCM-resistant Rho mutants and compared with the wild-type and the His-tagged wild-type Rho proteins (Table I). The Km for ribo(C)10 was 2 μM for the wild-type and the His-tagged wild-type Rho protein, whereas S266A (Fig. 2A) and S266Y gave a Km of 4 and 2 μM, respectively. Significantly, the Km values for M219K and G337S were both <0.1 μM (Fig. 2B) indicating that these proteins have an appreciably higher affinity for ribo(C)10 than does wild-type Rho. The S266Y mutation showed almost noncompetitive BCM inhibition kinetics with respect to ribo(C)10 with a Ki for bicyclomycin of 10 μM. This value is somewhat lower than that found for His-tagged wild-type Rho (Kd = 26 μM).

**Effects of the Mutations on Transcription Termination Activity—** In vitro Rho transcription termination was measured using a modified trp operon that contains a trp promoter and a Rho-dependent terminator (37, 45). Transcription termination is seen in the presence of Rho protein as shorter transcripts, compared with those seen in the absence of Rho. Fig. 3A shows the effects of the altered Rho proteins on transcription termination in the absence of BCM. Under identical conditions, wild-type Rho gave nearly complete transcription termination at the Rho-dependent site (bands B and C) with little read-through transcript (band A), the corresponding His-tagged wild-type, however, was 92% as effective in termination (Fig. 3A, lanes 2 and 3). The Rho-terminated transcripts from wild-type and His-tagged wild-type Rho showed a similar Rho-dependent ter-

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**Table II**

| Mutant | Poly(C) | Poly(U) | Poly(I) | Poly(A) |
|-------|--------|--------|--------|--------|
| Wild-type (wt) | 100 | 8.1 | 1.6 | 1.6 |
| His-tagged wt | 100 | 1.8 | 0.9 | 0.5 |
| M219K | 100 | 30.0 | 4.1 | 1.3 |
| S266A | 100 | <0.2 | <0.1 | 0.1 |
| G337S | 100 | 44.0 | 8.0 | 6.0 |
| S266Y | 100 | 2.0 | 0.2 | 0.4 |
mination pattern (Fig. 3A, lanes 2 and 3, bands B and C); however, the His-tagged protein gave an increased amount of an intermediate-size terminated transcript (band B). We have previously attributed these transcripts to a decreased rate of Rho tracking to the RNA polymerase (37).

M219K gave nearly the same ratio of Rho-terminated transcripts to read-through transcripts (Fig. 3A, lane 4, bands B and C) as the His-tagged wild-type Rho protein (Fig. 3A, lane 3), but S266A showed about a 50% decrease of Rho-terminated transcripts to wild type (Fig. 3A, lane 5, bands B and C). Furthermore, we observed a significant reduction in the percentage of shorter transcripts (band C) for both mutants. This shift in band composition was most noticeable for S266A (Fig. 3A, lane 5), where essentially all the transcripts were of intermediate size (band B). This suggests that the S266A mutation may alter the RNA tracking rate by decreasing the rate of ATP hydrolysis required for Rho translocation (14). We estimated the efficiency of Rho-dependent transcription termination for M219K and S266A to be 96 and 56%, respectively, of His-tagged Rho. Of the three mutants, G337S was the most efficient in terminating transcripts at the Rho-dependent site (Fig. 3A, lane 6).

**Fig. 2.** A, poly(dC)·ribo(C)10-stimulated ATPase activity for S266A (solid circles) and His-tagged wild-type Rho (solid squares) proteins. B, poly(dC)·ribo(C)10-stimulated ATPase activity for G337S (solid squares) and M219K (solid circles).

**Fig. 3.** A, transcription termination assays using the modified trp operon as a template comparing wild-type Rho, His-tagged wild-type Rho, M219K, S266A, and G337S Rho mutants. Lanes 1 and 7, the transcription assay without Rho; lane 2, wild-type Rho; lane 3, His-tagged wild-type Rho; lane 4, M219K; lane 5, S266A; and lane 6, G337S. The bands labeled A are the run-off transcript; bands B and C are Rho-terminated transcripts; and band D is a Rho-independent transcript. B, the effects of BCM on the transcription termination of M219K. C, the effects of BCM on the transcription termination of G337S.
It was equally as efficient at termination as the wild-type Rho and more so than the His-tagged wild-type Rho. We observed that the transcript termination pattern and the lengths of transcripts for G337S were comparable with those observed for wild-type Rho (Fig. 3A, lanes 2 and 6). A transcript termination pattern similar for wild-type Rho was also observed for S266Y (data not shown).

The effects of BCM concentration on transcription termination for M219K and G337S were determined. We observed a reduction in the intensities of both bands B and C compared with band A for M219K, with increasing BCM concentration (Fig. 3B, lanes 3–9). Analyzing the intensities of these bands provided an approximate $I_{50}$ value of 100 μM. Since the relative amounts of band C for this mutant was low, we observed no appreciable change in the band distribution when BCM concentration was varied. Furthermore, the intensity of band B did not increase with increasing BCM concentrations. Alternatively, G337S did show fewer Rho-dependent bands (bands B and C) as the BCM concentration was increased (Fig. 3C, lanes 3–10). An $I_{50}$ value of 180 μM was calculated for G337S, which is 36 times greater than the $I_{50}$ value obtained for wild-type Rho (37). Analyses of the band composition for G337S showed that with increasing BCM concentration the relative amounts of band B increased compared with band C (Fig. 3C); this mirrors the behavior of wild-type Rho (36). A similar study was not conducted with S266A because of the relatively low amounts of the shorter transcripts (Fig. 3A, lane 5, bands B and C) compared with the Rho read-through transcript (band A).

The Effects of BCM on Poly(C) Binding—It has been shown that BCM does not alter the binding of poly(C) to Rho (37), and gel mobility shift assays reveal that it does not interfere with Salmonella typhimurium HisG RNA binding to Rho (47). Likewise, we measured radiolabeled poly(C) binding to the mutant and wild-type Rho proteins in the presence and absence of BCM (1 mM). Fig. 4 shows the binding of poly(C) to the mutant His-tagged Rho proteins did not differ substantially from either wild-type or His-tagged wild-type Rho. The binding curves were nearly identical, and the 100% saturation levels were the same. Inclusion of BCM (1 mM) in the reaction did not affect poly(C) binding to Rho (data not shown). We concluded that the mutations themselves or BCM did not alter the extent of RNA binding to the primary site in any of the mutants.

**DISCUSSION**

The analysis of BCM inhibition of Rho has led to a model in which BCM interferes with the tracking of Rho at the secondary RNA-binding site (37) and the hydrolysis of ATP but not ATP binding (36). ATP hydrolysis is latent in Rho and requires RNA binding to both the primary and secondary binding domains (32). BCM inhibited ATP hydrolysis by a reversible, noncompetitive pathway with respect to ATP (36). Furthermore, BCM was shown to have a mixed-type inhibition with respect to RNA binding at the secondary RNA-binding (tracking) site (37). These data were in agreement with transcription termination reactions that showed intermediate-size transcripts (sizes between Rho terminated and run-off transcripts) when Rho was partially inhibited by the antibiotic. These transcripts are thought to arise from a slower tracking of Rho toward the elongation complex due to BCM binding. Slower Rho tracking rates lead to inhibition of transcription termination by not allowing Rho to catch the RNA polymerase. This kinetic coupling model predicts that slowing the tracking rate of Rho just below the rate of RNA polymerization would lead to complete inhibition of transcription termination while substantial Rho ATPase activity would remain (18). Previously, we reported that Rho transcription termination processes were more sensitive to bicyclomycin than the poly(C)-stimulated ATPase activity (37). Also, the $I_{50}$ values for bicyclomycin inhibition of RNA-stimulated ATPase activities are greater with RNAs that are stronger activators of Rho ATPase activity (37). We have attributed the differences in the $I_{50}$ values in part to the different sensitivities of the Rho-RNA complexes toward BCM. We found that the $I_{50}$ value for BCM in the poly(U)-dependent ATPase assay ($I_{50} \approx 10 \mu M$) was lower than that observed in the poly(C)-dependent ATPase assay ($I_{50} \approx 60 \mu M$). Poly(U) is a weaker activator of ATPase activity than poly(C) (25). Also, oligoribonucleotides, ribo(U)nCm, varying in U and C composition, affect the $K_m$ of binding at the secondary site and the $V_{max}$ of Rho ATPase activity (48). These results suggest that poly(U) binds weaker to the secondary site of Rho than poly(C) and that decreased levels of BCM are required to inhibit Rho-mediated processes that employ poorer ATPase-activating RNA substrates. Consistent with this notion, the $trp t'$ is a weaker activator of Rho ATPase activity than poly(C) (49). The data presented suggest that two factors account for the observed BCM inhibition of Rho-mediated ATP hydrolysis. First is that BCM binds to Rho and competes with the RNA binding at the secondary site leading to inhibition of transcription termination. This process also inhibits ATP hydrolysis by preventing the RNA-induced conformational changes necessary for hydrolysis (37). The second factor is that BCM binds at or near the ATP catalytic site and blocks formation of the active conformation needed for hydrolysis. These two factors imply that the ATP catalytic
domain is in proximity to the secondary RNA-binding site.

Rho protein can be divided into two distinct functional domains (16, 30, 50), the primary RNA binding domain (20, 21, 46) and the ATP binding domain (31, 24, 51). The primary RNA-binding site has been located within the N-terminal 130 amino acids with a conserved RNA binding motif at residues 61–64 (20, 27), and the ATP binding and hydrolysis domain has been located from residue 131 to the C-terminal of Rho (27, 31). A secondary RNA binding domain exists but is less characterized and is only defined as point mutations affecting poly(dC)-ribo(C)10-stimulated ATPase activity (29, 33).

Sequence alignment among the E. coli Rho protein and the α- and β-subunits of both E. coli and bovine F1-ATP synthase have identified nearly identical ATP binding and hydrolysis domains (28, 29). Our sequence alignment for these five proteins (data not shown) is similar to the sequence and secondary structural alignments that have produced models of Rho tertiary structure (28, 29, 52) similar to bovine F1-ATP synthase (53). The secondary structure elements in the β-subunit of F1-ATP synthase was divided into three domains: the N-terminal β-barrel (sheets α–f), the central nucleotide binding domain (helices A–I and sheets 1–9), and the C-terminal domain (helices 1–6) (53). Table III summarizes our comparisons of the Rho secondary sequence predictions with that of the bovine β-subunit of F1-ATP synthase, which are relevant to the ATP hydrolysis pocket and the predicted BCM binding domain. We have maintained the helix and sheet numbering system introduced for the bovine F1-ATP synthase (53).

The initial alignment of the GKT residues from Rho (residues 183–185) to the corresponding residues in the ATP hydrolysis pocket of the β-subunit of F1-ATP synthase (residues 161–163) allowed the N-terminal 200 amino acids of Rho to be aligned. The sequence of the first 159 residues (E. coli Rho

![Projected rho monomer structure showing the bicyclomycin binding pocket](image)

Fig. 5. A MolScript (53) model of the proposed Rho structure based on the F1-ATP synthase structure. This model shows the α-helices and β-sheets involved in the ATP hydrolysis pocket. Critical residues that correspond to alterations leading to BCM resistance are indicated and drawn as a ball and stick model. Residue Lys-181, the site of reductive amination of the BCM aldehyde derivative, is also shown. The BCM binding pocket is projected to be within the dashed box.

| Protein | Helix B | Helix C | Sheet 1 | Sheet 2 | Sheet 3 | Sheet 4 |
|---------|---------|---------|---------|---------|---------|---------|
| Rho     | 183–197 | 214–226 | 203–209 | 227–233 | 259–268 | 311–317 |
| F1-ATPase | 161–173 | 189–202 | 179–187 | 213–221 | 250–258 | 303–311 |

Identifying the Bicyclomycin Binding Domain

TABLE III

The alignment of bovine F1-ATP synthase β-subunit secondary structure to Rho relevant to the ATP hydrolysis pocket and the BCM binding domain
numbering) did not exhibit sequence similarity. Alignment of the N-terminal region, however, was less critical because of the different functions of Rho (RNA binding) and F$_1$-ATP synthase. A structural model of the N-terminal domain based on x-ray crystallization and NMR studies on this fragment has been presented (21, 22). Sequence similarity became noticeable at position 160 with the sequence RVLDLA. The first gap after Rho residue 200 is seen as an extension of the bovine $\beta$-subunit, which is a loop region between helix B and sheet 1. All gaps over five residues appear to occur at the turn regions away from the catalytic domain. If the Rho sequence is aligned with just the E. coli $\beta$-subunit F$_1$-ATP synthase there are no large gaps near the catalytic domain. The only other gap in the Rho sequence occurs after residue 383 in the C-terminal domain. This gap corresponds to two $\alpha$-helical segments thought to interact with the $\gamma$-subunit of the F$_1$-ATP synthase complex (53). Alignment of the Rho RKI residues 388–390 to those in the $\beta$-subunits (residues 408–410) on the C-terminal side of the gap was used to position the last 45 residues of Rho to the end of the F$_1$-ATP synthase subunits where part of the ATP binding domain is located (53).

Our model of Rho is similar to that reported (24, 29), except that the C-terminal helices 1 and 2 in the F$_1$-ATP synthase (residues 364–405 in the $\beta$-subunit) are absent. A MolScript (54) model of Rho is presented in Fig. 5 in which the helices and sheets that surround the ATP binding pocket are blue and green, respectively. The residues corresponding to those that produce BCM resistance and that are covalently linked to the green, sheets that surround the ATP binding pocket are presented in Fig. 5 in which the helices and sheets are defined (53). These secondary structures are defined as reported; however, the corresponding amino acid positions were determined by examination of the bovine $\beta$-subunit structure, as defined in Table III. The BCM-resistant mutants have amino acid alterations that map to the catalytic site of ATP hydrolysis. These mutants are D210A (55), M219K, S266A, and G337S (10). We have also reported that an aryl aldehyde derivative of BCM can inhibit Rho transcription termination in a manner similar to BCM and can lead to permanent inactivation of Rho upon addition of NaBH$_4$ (43, 44). Significantly, inclusion of BCM in these reactions led to successively lower levels of irreversible Rho inhibition after dialysis, showing that BCM binding was competitive with the aryl aldehyde (43). The side of addition was Lys-181, with an approximate stoichiometry of one adduct per two subunits (44). Lys-181 corresponds to Gly-159 in the $\beta$-subunit of F$_1$-ATP synthase and is near the catalytic ATP hydrolysis site (31).

Our studies on the kinetic properties of BCM-resistant mutants coupled with structural information obtained from the closely homologous bovine F$_1$-ATPase structure has provided added insights into the possible locations of the secondary RNA and BCM binding domains in Rho. Both M219K and G337S have decreased $K_m$ values for ribo(C)$_{10}$, indicating that these proteins bind RNA tighter than wild-type Rho at the secondary site. Furthermore, BCM competes with ribo(C)$_{10}$ for binding to Rho at the secondary site (37). Gly-337 is next to other residues that have been implicated in the secondary RNA binding domain by analysis of other mutants (29) and can explain the competition between RNA and BCM. Moreover, we found that G337S has a “super Rho” phenotype (56–58), terminating in vitro transcription better than His-tagged wild-type Rho. Correspondingly, M219K when mapped to the F$_1$-ATP synthase structure is found in helix C on the surface of Rho across from Gly-337. The spatial distance separating Met-219 and Gly-337 on an individual subunit is longer than expected for each residue to be part of the same secondary RNA binding domain. The secondary binding site could be close to the interface of two Rho subunits comprising Met-219 from one subunit and Gly-337 from the adjacent subunit assembled in a $C_2$ symmetry (29, 59). The locations of these residues on the F$_1$-ATP synthase model do not preclude this speculation.

S266A neither showed significant changes in the $K_m$ value for ribo(C)$_{10}$ nor changes in the ratio of poly(U) to poly(C) upon RNA activation of the ATPase activity compared with the His-tagged wild-type Rho. In addition, tyrosine substitution at Ser-266 restored BCM sensitivity and drug binding. The Ser-266 position on the Rho structural model (Fig. 5) is located at the entrance to the deep cleft defined by the four $\beta$-sheets and two $\alpha$-helices at the catalytic site for ATP hydrolysis. We project that this region defines the BCM binding pocket. This suggests the serine hydroxyl group at position 266 is important for drug binding. Similarly, the D210G BCM-resistant mutant reported by Nowatzke et al. (55) is predicted to be in this locale.

These studies suggest that BCM binds within the deep cleft in Rho that makes up the catalytic site for ATP hydrolysis. The BCM binding pocket likely partially overlaps the extended secondary RNA binding domain. We suggest that drug binding competes with RNA binding and further disrupts the protein conformational change required for ATP hydrolysis. Studies are in progress testing this structural theory and defining the stoichiometry and regional binding distribution of BCM and ATP in Rho.

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