The Antibiotic Bicyclomycin Affects the Secondary RNA Binding Site of *Escherichia coli* Transcription Termination Factor Rho*

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The interaction of Rho and the antibiotic bicyclomycin was probed using *in vitro* transcription termination reactions, poly(C) binding assays, limited tryptic digestions, and the bicyclomycin inhibition kinetics of ATPase activity in the presence of poly(dC) and ribo(C)10. The approximate *I*_50 value for the bicyclomycin inhibition of transcription termination at Rho-dependent sites within a modified *trp* operon template was 5 μM. At antibiotic concentrations near the *I*_50 value, bicyclomycin inhibition of Rho-dependent transcripts was accompanied by the appearance of a new set of transcripts whose size was midway between the Rho-dependent transcripts and the readthrough transcripts. Bicyclomycin did not inhibit poly(C) binding to Rho. In the presence of poly(dC), bicyclomycin showed a reversible mixed inhibition of the ribo(C)10-stimulated ATPase activity. The extrapolated *K*_f for bicyclomycin was 2.8 μM without ribo(C)10 and increased to 26 μM in the presence of ribo(C)10. Correspondingly, the *K*_m(app) for ribo(C)10 without bicyclomycin was 0.8 μM and with bicyclomycin was 5 μM at infinite inhibitor concentration. The data suggested that the antibiotic binds to Rho, influencing the secondary RNA binding (tracking) site on Rho and slows the tracking of Rho toward the bound RNA polymerase.

The structurally unique antibiotic bicyclomycin (Scheme 1) (1, 2) has been shown to target a broad spectrum of Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella*, *Salmonella*, *Shigella*, and *Citrobacter* (1–3). The primary site of action for bicyclomycin was shown to be Rho transcription termination factor in *E. coli* (4, 5). DNA coding for Rho protein from antibiotic-resistant mutants was able to confer drug resistance to otherwise sensitive cells (4). Bicyclomycin has been shown to inhibit Rho-poly(C)-dependent ATPase activity with simple noncompetitive kinetics with respect to ATP (6).

Rho protein is composed of six identical 46-kDa proteins of 419 amino acids (7) in a proposed planar, hexagonal, geometric D3 symmetry (8–10). Rho transcription termination factor is required to stop transcription at several Rho-dependent termination sites, including the proximal region of the lacZ cistron (11), the AtRII terminator (12), and the *trp* t′ at the end of the tryptophan operon in *E. coli* (13).

ATPase and helicase activities associated with Rho are essential for transcription termination (14–16). The ATPase activity is latent until RNA binds to Rho. RNA binding to both a primary and a secondary site is required to stimulate ATPase activity. Reports have proposed a tethered tracking mechanism in which, initially, Rho binds tightly to RNA at a rut site and tracks toward the stalled RNA polymerase using the secondary RNA-binding site (for reviews, see Refs. 15 and 16). According to this mechanism, RNA binds tightly to the primary site in Rho and remains bound during the Rho transcription termination process, but this binding is not sufficient to stimulate ATPase activity (17, 18). RNA binding to the secondary or tracking site causes further change in the structure of Rho, which stimulates an ATPase activity (17, 19). A 5′ to 3′ DNA:RNA helicase activity (20) is likely to be required for transcription termination.

Primary RNA and ATP binding domains of Rho have been determined (21–24). The N-terminal 151 amino acids bound *trp* t′ and poly(C) (23) and the first 116 amino acids to ribo(C)10 (25). Mutants that affect primary binding have been located within the first 116 amino acids (26, 27), and mutagenesis of residues 62 and 64 decreased RNA binding activity (28). The ATP binding domain extends from residues 160 to 184 and contains sequence similarity to *E. coli* F1 ATPase α and β subunits and adenylyl kinase (23). Mutations at residues 181, 184, and 265 affected ATP hydrolysis without changing RNA binding to the primary site (21). At present, little is known about the secondary RNA binding site. The Rho mutant suA1 has been identified as a secondary site mutant with a single amino acid change at residue 352. This finding suggests that this region of the sequence is involved in Rho tracking (29, 30).

A working model of the mechanism of Rho transcription termination relies on the coupling of RNA binding, at both primary and secondary binding sites, to ATP hydrolysis, promoting RNA tracking, helicase activity and eventual transcription termination (14, 31). Mutations that conferred bicyclomycin resistance (M218K, S266A, G337S) were located in the ATP binding domain (4); however, kinetic studies demonstrated that bicyclomycin was a reversible, simple, noncompetitive inhibitor of Rho with respect to ATP (6). To examine the effects of bicyclomycin on Rho activities further, *in vitro* transcription termination assay, poly(C) binding, and poly(dC)-ribo(C)10-stimulated ATPase assays were carried out. We report, herein, that bicyclomycin inhibits *in vitro* Rho transcription termination processes. Evidence is presented that the antibiotic affects the RNA binding to the secondary site in Rho and modifies the rate of RNA tracking.

**EXPERIMENTAL PROCEDURES**

*Materials and Enzymes—*Bicyclomycin was purified by three successive silica gel chromatographies using 20% methanol/chloroform as the eluant, as described (6). Dihydrobicyclomycin was prepared by catalytic hydrogenation of bicyclomycin (32). Rho protein was isolated from *E. coli* AR120 containing the overexpressing plasmid p39-AS (33) following previously published protocols (33). Rho purity was determined by
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SDS-PAGE, and concentrations were determined according to the Lowry method (34). T4 polynucleotide kinase was purchased from Promega Co. (Madison, WI). [γ-32P]ATP and [α-32P]UTP (3000 Ci/mmol) were purchased from DuPont NEN; nucleotides and RNase inhibitor were from Ambion, Inc. (Austin, TX), polynucleotides were purchased from Pharmacia Biotech Inc., and ribo(C)10 was purchased from Oligos Etc. (Wilsonville, OR). 1-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was obtained from Sigma. HA-T7 nitrocellulose filters used for ligating assays were obtained from Millipore Co. (Bedford, MA), and polyethyleneimine thin-layer chromatography plates used for ATPase assays were purchased from J. T. Baker, Inc. All other chemicals were reagent grade.

In Vitro Transcription Termination Assay—E. coli RNA polymerase was purified according to the method of Burgess and Jendrisak (35), with minor modification. The Bio-Gel A-5m column was replaced with a Sephacryl S4000 column. The DNA substrate was a truncated form of the trp operon (13). The trpIII-Sall fragment from a pWU5 plasmid was ligated into the EcoRI-Sall sites of pGEM5 resulting in pTRPS, and the isolated BamHI-Sall fragment from pTRP5 was used as the template for the assay.

In Vitro transcription was carried out in a 10-μl volume containing 20 mM Tris acetate, pH 7.9, 150 mM KCl, 4 mM magnesium acetate, 0.1 mM DTT, 0.1 mM EDTA, 200 μM each of ATP, CTP, and GTP, 20 μM UTP, 7 μCi of [γ-32P]UTP, 0.1 μCi of DNA template, 0.4 μM Rho, 0.01 μg/ml E. coli RNA polymerase, and 70 nM Rho protein with either bicyclomycin (0.5–100 μM) or dihydrobicyclomycin (5–100 μM). The samples were incubated at 37°C (20 min), diluted with 100 μl of 0.02 M sodium acetate, 1 mM EDTA, and carrier RNA to 8.0 μg/ml, extracted with phenol, precipitated with ethanol, and dissolved in loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromphenol blue, and 2 mM EDTA). The RNA products were separated on a 5% polyacrylamide, 8 M urea gel and visualized using autoradiography. The relative amounts of radioactive incorporation of each band were determined using densitometry.

RNA 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. Reaction mixtures were incubated at 37°C (30 min) and then 70°C (10 min) and loaded on polyacrylamide gels. The desired length of oligomer (approximately 130 nt) 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 Mgl2, 0.1 mM EDTA, 0.1 mM DTT) and 0–113 nM Rho in a total volume of 100 μl. Two procedures were employed. First, bicyclomycin (400 μM) or dihydrobicyclomycin (1.5 mM) was added to the Rho solution after it was prerun in H2O and dried. [γ-32P]UTP, as described previously (13), and visualized using autoradiography (Fig. 1). Addition of 10 μl of stop buffer (6 x 50 mM each of ATP, CTP, GTP, and UTP) 8.0, 60 mM KCl, 5 mM MgCl2, 0.1 mM DTT) was used as a control for the reaction. The mixture was electrophoresed in 15% urea-SDS-PAGE at 120 V for approximately 8 h. The proteins were visualized using Coomassie Blue R-250. One microliter of 1 μl poly(C), 1 μl of 3.2 μM poly(C), 1 μl of 0.2 μl ribo(C)10, 1 μl of 10 mM ATP, and 1 μl of 4 mM bicyclomycin were added to the appropriate samples in a total volume of 10 μl.

Poly(C)-Riboc(C)10-dependent ATPase Activity—ATPase activity assays were carried out in 20-μl reactions containing 30 mM Tris acetate, pH 7.9, 0.4 mM magnesium acetate, 50 mM potassium acetate, 10 mM DTT, 0.2 mM ATP, 0.015 μCi of [γ-32P]ATP, 3.2 μl poly(C) (average size, 300 nt), ribo(C)10 (0.7–14 μM), 18 nM Rho, and with either bicyclomycin (0–20 μM) or dihydrobicyclomycin (0–40 μM). Reactions were preincubated at 32°C for 2 min prior to the addition of ATP. Aliquots (2 μl) were removed at various times (15, 30, 45, 60, 75, and 90 s) during the reaction and spotted onto polyethyleneimine TLC sheets that had been prerun in H2O and dried. [γ-32P]ATP and [32P]UTP, were separated by chromatography on the polyethyleneimine sheets using 0.75 M KH2PO4, pH 3.5, as the mobile phase and then located by autoradiography. The radioactive spots were either cut out and counted by liquid scintillation, according to published methods, or the developed TLC plates were cut into 1-ml strips, dried, and scanned using a Fuji BAS 1000 Bio Imaging Analyzer and analyzed using the Macintos PAS analysis program. The initial rates of reactions were determined by plotting the amount of ATP hydrolyzed versus time.

RESULTS

Inhibition of Rho Transcription Termination by Bicyclomycin and Dihydrobicyclomycin—The ability of Rho to terminate in vitro transcription processes using a modified trp operon was first reported by Platt and co-workers (13). As we have diagrammed, Platt found that the read-through (run-off) transcript from the 800-base pair BamHI-SalI fragment starting from the trp p promoter consists of 545 nt (Band C) (see Fig. 1). Two terminators are present on this template DNA, a Rho-independent terminator in which approximately 25% of the message terminates at 245 nt (trp t, Band A) and a Rho-dependent terminator at 470 nt (trp t′, Band B).

E. coli polymerase transcriptional processes were monitored by the incorporation of [α-32P]UTP, as described previously (13), and visualized using autoradiography (Fig. 1). Addition of Rho to the reaction led to the Rho-terminated transcript at trp t′ (Fig. 1, lane 2, Band B) whereas the 545-nt read-through transcript was observed when Rho was not present (Fig. 1, lane 1).
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1, Band C). Inclusion of bicyclomycin with Rho-containing solutions inhibited Rho-dependent termination processes (Fig. 1, lanes 3–11). The approximate I50 value for bicyclomycin was 5 μM, where the I50 value corresponded to the amount of bicyclomycin that gave 50% of the transcript terminated at the trp t’ site.

There were two interesting findings for bicyclomycin inhibition of Rho transcription termination. First, the amount of bicyclomycin used to inhibit transcription termination reactions (I50 ∼ 5 μM) was more than 10 times less than the amount needed to inhibit Rho-poly(C)-dependent ATPase activity (I50 = 60 μM) (4). We have attributed the difference in the I50 values in these two assays, in part, to the different sensitivities of the Rho-RNA complexes toward bicyclomycin. We found that the I50 value for bicyclomycin in the poly(U)-dependent ATPase assay (I50 = 10 μM)2 was lower than that observed in the poly(C)-dependent ATPase assay (I50 = 60 μM). Poly(U) is a weaker activator of ATPase activity than poly(C) (17). Also, oligoribonucleotides, ribo(U7C9)n, varying in U and C composition, affect the Kₘ of binding at the secondary site and the Vₘₐₓ of Rho ATPase activity (19). The Kₘ increased and the Vₘₐₓ decreased when an increasing number of ribo(U)s was placed at the 5’ site of the octoribonucleotide. These results suggest that poly(U) binds weaker to the secondary site of Rho than poly(C) and that decreased levels of bicyclomycin 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) (21).

Second, we found appreciable amounts of a new set of intermediate-size transcripts when bicyclomycin concentrations close to the observed I50 value were used (Fig. 1, lanes 5–8, Band D). These transcripts were not observed at low or high bicyclomycin concentrations. The detection of these transcripts was consistent with the notion that bicyclomycin concentrations near the I50 value sufficiently slowed down but did not totally abolish the Rho tracking process. Significantly, in order for transcription to effectively terminate at the trp t’ site, the rate at which Rho transverses 5’ to 3’ toward the stalled polymerase must be faster than the rate at which the polymerase proceeds through the stall site on the DNA. If either the rate of the polymerase is increased or the rate of Rho translocation is decreased, termination would not happen. These results are consistent with the notion that bicyclomycin may affect the Rho secondary RNA binding site and slow the rate of tracking toward the polymerase. This phenomenon leads to the production of a new set of transcripts that is somewhat larger than 470 nt in length.

A similar set of experiments was conducted with dihydrobicyclomycin (Scheme 1). Dihydrobicyclomycin is a bicyclomycin analog in which the C(5)-C(5a) exomethylene group has been reduced. We observed that dihydrobicyclomycin inhibited Rho transcription termination and that the approximate I50 value was 20 μM (data not shown). The increase in the I50 value for dihydrobicyclomycin compared with bicyclomycin in the transcription termination assay was similar to the observed differ-

2 A. Magyar and W. R. Widger, unpublished results.

FIG. 2. Filter binding data of 25 nM labeled poly(C) binding to Rho transcription termination factor without bicyclomycin (○), with 400 μM bicyclomycin (●), with 5 mM ATP (△), and with 5 mM ATP plus 400 μM bicyclomycin (□). The percentage of 32P-labeled poly(C) retained on pretreated nitrocellulose filters was plotted as a function of Rho protein concentration. Each point represents the average of two parallel determinations.

Bicyclomycin and Dihydrobicyclomycin Inhibition of Rho-Poly(dC)-Ribo(C)₁₀-stimulated ATPase Activity—The effects of bicyclomycin on Rho-dependent transcription termination suggest that bicyclomycin may affect the secondary RNA binding (tracking) site. The secondary binding site can be distinguished from the primary binding site (racing binding site) with kinetics using poly(dC) and ribo(C) (18). Poly(dC) binds to Rho at the primary binding site without stimulating ATP hydrolysis. Upon further addition of ribo(C)₇₋₁₀ the ATPase activity is
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stimulated. Fig. 4A shows the effects of bicyclomycin concentration on the poly(dC)-ribo(C)10-stimulated ATPase activity. We observed that increasing concentrations of bicyclomycin decreased the rate of ATP hydrolysis. The kinetics showed mixed inhibition when 1/V was plotted against 1/[ribo(C)10].

The binding of ribo(C)10 is dependent on the amount of bicyclomycin present. The $K_{m,app}$ for ribo(C)10 without bicyclomycin was 0.8 $\mu$M. This value was lower than that previously observed (30, 37, 38). The extrapolated $K_{m,app}$ for ribo(C)10 was 5 $\mu$M at infinite bicyclomycin concentration. This increase in the $K_{m,app}$ for ribo(C)10 indicated that bicyclomycin influenced the ribo(C)10 binding. A $K_i$ value of 2.8 $\mu$M, the dissociation equilibrium constant for the interaction of bicyclomycin with the free enzyme (Rho plus poly(dC) without ribo(C)10), was determined from the plot of the slopes versus bicyclomycin concentration (Fig. 4B) derived from Fig. 4A. The corresponding $K_i$ value in the presence of ribo(C)10 was 26 $\mu$M, calculated from a plot of the intercepts (1/V$_{max}$) versus bicyclomycin concentration (Fig. 4B). This value was comparable with the $K_i$ value of 20 $\mu$M previously determined for ATP hydrolysis in the presence of poly(C) (6).

Dihyrobicyclomycin was also used as an inhibitor of Rho-poly(dC)-ribo(C)10-stimulated ATPase activity and gave a similar kinetic profile. The double-reciprocal plot is provided in Fig. 5A. The $K_{m,app}$ for ribo(C)10 without dihyrobicyclomycin was 0.9 $\mu$M while the extrapolated $K_{m,app}$ for app for ribo(C)10 was 3.3 $\mu$M at infinite dihyrobicyclomycin concentration. Values for $K_i$ with and without ribo(C)10 were 45 and 10 $\mu$M, respectively (Fig. 5B). These results indicated that both bicyclomycin and dihyrobicyclomycin displayed comparable inhibitory properties in the ATPase assay when poly(dC)-ribo(C)10 was used in place of poly(C). The $K_i$ for dihyrobicyclomycin without ribo(C)10 was about 4 times higher than the corresponding value for bicyclomycin. This difference in activities for these two compounds was similar to the differences previously observed in the $I_50$ values for Rho-dependent transcription termination and the $K_i$ values for inhibition of poly(C)-dependent ATPase activity.

DISCUSSION

In vitro Rho-dependent transcription termination reactions were inhibited by bicyclomycin with an $I_50$ value of $5 \mu$M (Fig. 1). At these concentrations, intermediate-size transcripts midway in length between the Rho-terminated and the readthrough transcripts were seen. This observation suggested bicyclomycin interfered with the tracking rate at which Rho binds to RNA at the secondary site and translocates down the template. A slower tracking rate would permit the polymerase to move through the stall site before transcription termination. The continued synthesis of RNA would lead to transcripts of intermediate length provided Rho could catch the polymerase before the end of the template. This kinetic coupling mechanism has been used to explain mutations in the $\beta$ subunit of RNA po-
The hypothesis that bicyclomycin affects the secondary RNA binding (tracking) site is supported by several additional observations. We found that the percent of poly(C) bound to Rho could catch up and effectively terminate transcription (39). Kinetic complementation was suggested over a direct contact between Rho and the RNA polymerase. The mutation in the $\beta$ subunit of RNA polymerase slowed down the polymerase rate so that a slowly tracking mutant of Rho could catch up and effectively terminate transcription (39).

Our findings showed that dihydrobicyclomycin displayed properties similar to bicyclomycin in the transcription termination, Rho-poly(C) binding, and poly(dC)-ribonucleotide-stimulated ATPase assays. These results indicated that bicyclomycin expressed its inhibitory activity in in vitro Rho-mediated processive transcription principally by a noncovalent binding process. This observation is important since bicyclomycin readily reacts with nucleophiles at the C(5)-C(5a) exomethylene group to give covalent adducts (40, 41). Reduction of this structural unit in nucleophiles at the C(5)-C(5a) exomethylene group to give covalent adducts (40, 41).
bicyclomycin.

Recently, several laboratories have demonstrated that Rho-mediated processes were sensitive to bicyclomycin. Transcription termination reactions catalyzed by a Rho isolated from a Gram-positive bacterium Micrococcus luteus were sensitive to bicyclomycin (42). Also, bicyclomycin-resistant mutants were localized to rho and rpoB (4, 43). These findings are consistent with the notion that bicyclomycin slows the tracking rate of Rho from the rut (binding) site toward the stalled RNA polymerase because a reduction in the transcription rates would allow a more slowly moving Rho to catch up to the polymerase and terminate transcription. Our results, however, do not rule out that additional factors (e.g. NusG) (43–46) that control the rate of Rho transcription termination could also influence bicyclomycin resistance in E. coli.

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