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Effects of Bicyclomycin on RNA- and ATP-Binding Activities of Transcription Termination Factor Rho

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Bicyclomycin is a commercially important antibiotic that has been shown to be effective against many gram-negative bacteria. Genetic and biochemical evidence indicates that the antibiotic interferes with RNA metabolism in *Escherichia coli* by inhibiting the activity of transcription termination factor Rho. However, the precise mechanism of inhibition is not completely known. In this study we have used in vitro transcription assays to analyze the effects of bicyclomycin on the termination step of transcription. The Rho-dependent transcription termination region located within the *hisG* cistron of *Salmonella typhimurium* has been used as an experimental system. The possible interference of the antibiotic with the various functions of factor Rho, such as RNA binding at the primary site, ATP binding, and hexamer formation, has been investigated by RNA gel mobility shift, photochemical cross-linking, and gel filtration experiments. The results of these studies demonstrate that bicyclomycin does not interfere with the binding of Rho to the loading site on nascent RNA. Binding of the factor to ATP is not impeded, on the contrary, the antibiotic appears to decrease the apparent equilibrium dissociation constant for ATP in photochemical cross-linking experiments. The available evidence suggests that this decrease might be due to an interference with the correct positioning of ATP within the nucleotide-binding pocket leading b an inherent block of ATP hydrolysis. Possibly, as a consequence of this interference, the antibiotic also prevents ATP-dependent stabilization of Rho hexamers.

Bicyclomycin (Bicozamycin), a natural compound obtained from cultures of *Streptomyces sapporonensis*, is a structurally unique antibiotic that has been shown to be effective against gram-negative bacteria such as *Escherichia coli*, *Klebsiella*, *Salmonella*, *Shigella*, and *Citrobacter* (37) and the gram-positive bacterium *Micrococcus luteus* (22, 23). It is a relatively weak antibiotic, with MICs ranging between 82 and 164 μM for various sensitive strains of *E. coli*, and its current principal application is as a feed additive for livestock (37). The mechanism of action of bicyclomycin is also distinct from those of other known classes of antibiotics. It has been theorized that its function is associated with the covalent attachment of a nucleophilic protein residue (cysteine, histidine, or lysine) to the C-5–C-5a exomethylene group of the antibiotic (37).

Both genetic and biochemical evidence indicates that the primary site of bicyclomycin action in *E. coli* is the transcription termination factor Rho. The first genetic evidence of a direct interaction between bicyclomycin and the transcription termination factor Rho was obtained by Zwiefka et al. (39). UV-generated bicyclomycin-resistant *E. coli* mutants have been shown to have mutated *rho* alleles (39). DNA coding for these mutant *rho* genes was able to confer antibiotic resistance to otherwise sensitive cells (39). Other mutations conferring drug resistance to *E. coli* have been mapped in the *rho* gene (38). More recently, an *M. luteus* bicyclomycin-resistant mutant harboring a missense mutation in the *rho* gene has been isolated, with the mutation changing an evolutionarily conserved Asp^{474} residue in the ATP-binding domain of the protein (23). There is also biochemical evidence of a direct Rho-bicyclomycin interaction. Bicyclomycin directly affects the poly(C)-dependent ATPase activity of Rho (39). Prolonged incubation of the drug with Rho, without RNA, gave rise to Rho-bicyclomycin-substituted adducts with diminished transcription termination activities (25). More recently, the functional groups of the molecule have been defined in order to synthesize bicyclomycin-derivative photoaffinity reagents and to identify the bicyclomycin-binding domain on Rho (25, 26, 34). These findings place bicyclomycin, dihydrobicyclomycin, and their semisynthetic derivatives (37) in a category with rifamycin B and actinomycin D, two other antibiotics known to interfere with RNA metabolism.

Rho-dependent transcription termination is a key event in a variety of metabolic processes. In gram-negative bacteria Rho is essential for cell growth and is responsible for the phenomenon of polarity, transcriptional attenuation, transcription termination at the end of gene clusters, and preventing the synthesis of unused transcripts during conditions of physiological stress (1, 2, 19, 28, 30, 32). It has been reported that noninhibitory concentrations of bicyclomycin increase basal-level expression of the *tna* operon as a consequence of suppression of Rho-dependent transcription termination at the level of the leader region and relieve polarity in the *trp* operon of *E. coli* (38).

The Rho protein is a homohexamer with a pseudo-D3 symmetry comprising three asymmetric dimers (14). Each monomer has specific domains for the binding of RNA and ATP (11, 31). According to a current model, Rho first binds to an entry site in the 5′ proximal region of the nascent RNA and then translocates unidirectionally along the RNA in a reaction coupled with the hydrolysis of ATP (27, 28, 35). When the Rho

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translocation rate exceeds that of the elongating RNA polymerase, it presumably catalyzes the release of the RNA and the dissociation of the elongation complex with the aid of its RNA-DNA helicase function. This model is substantiated by kinetic analysis of the RNA-dependent ATPase activity which shows that Rho has two functionally, albeit not physically, distinguishable sites for its interactions with RNA (29). One site, termed the primary site, can bind with a high-affinity and in an ATP-independent manner to single-stranded RNA spanning at least 70 nucleotides with a characteristic requirement for a high cytosine content and a low guanosine content (3, 28). The other site, termed the secondary site, specifically interacts with a low affinity with short RNA segments up to eight nucleotides in a manner coupled with binding and hydrolysis of ATP (28).

Although several studies have provided information concerning the target of bicyclomycin, they have not completely revealed either the precise mechanism of Rho inhibition by the antibiotic or the site and functional domain(s) where bicyclomycin binds to the protein. Kinetic in vitro studies of the poly(C)-dependent ATPase activity of Rho indicate that inhibition occurs by a rapid and reversible binding of bicyclomycin to Rho which is noncompetitive with respect to ATP (24). More recently, while the present work was in progress, the results of bicyclomycin inhibition kinetics studies of ATPase activity in the presence of both poly(dC) and poly$(C)_{10}$ have suggested that the antibiotic influences the secondary binding site on Rho and slows the tracking of Rho toward the RNA polymerase (18).

In the present work the molecular mechanism by which bicyclomycin blocks the activity of Rho has been further investigated. The effect of the antibiotic on the termination step of transcription has been analyzed by in vitro transcription experiments with the Rho-dependent termination region located within the *hisG* cistron of the *Salmonella typhimurium his* operon (3, 9). The same substrate has been also used in RNA gel mobility shift experiments in an attempt to detect the possible interference of bicyclomycin with RNA binding and the activation of Rho monomers at the primary site. The kinetics of ATP binding to Rho in the presence of various concentrations of the antibiotic were measured by UV cross-linking titration experiments. Finally, gel filtration experiments have been used to analyze the possible interference of bicyclomycin on the formation of Rho hexamers under certain experimental conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmid pGEM294 was obtained by cloning a 294-bp *Hin*dII-*Sau*3AI DNA fragment containing the central region of the *hisG* cistron of *S. typhimurium* into the *Sma*I-*Bam*HI sites of vector pGEM3Z (Promega, Madison, Wis.). Plasmid pHG360 has been described previously (3). Strain DH5α[F^- Φ80d *lacZ*ΔM15 *endA1 recA1 hsdR17 supE44 thi*-1 d^- *gyrA96* Δ(*lacZYA-argF*) *U169*] was used in the cloning procedures. The rich medium used in the study was Luria-Bertaini broth (20) supplemented with 50 μg of ampicillin per ml when required.

DNA procedures. DNA fragments were isolated through acrylamide slab gels and were recovered by electroelution as described previously (33). The 3′ end labelling was performed with the Klenow fragment of DNA polymerase (5). Electrophoretic strand separation of DNA fragments was carried out as described by Sambrook et al. (33).

S1 nuclease mapping. RNA-DNA hybridization, S1 nuclease digestion, and analysis of the hybrids on polyacrylamide denaturing gels were performed as described by Favaloro et al. (12) under the same conditions described previously (6).

Quantitative analysis of the different transcripts was performed by densitometry with a Scanmaster 3 (Howtek, Inc., Hudson, N.H.), a high-performance desktop flat-bed color scanner equipped with the RFLPrint (Pdi, Huntington Station, N.Y.) software package, or by directly counting the radioactivity bands with a PhosphoImager SI imager (Molecular Dynamics, Inc., Sunnyvale, Calif.).

In vitro transcription. Supercoiled plasmid DNAs were transcribed in vitro by the following procedure. The reaction mixture (50 μl), containing 0.5 pmol of DNA template, 1 μg of purified *E. coli* RNA polymerase holoenzyme (Boehringer Mannheim GmbH, Mannheim, Germany), and, when present, 100 nM *E. coli* Rho protein (kindly provided by B. Stitt), was preincubated for 5 min at 37°C in a solution of 20 mM Tris acetate (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 4 mM magnesium acetate, 50 mM KCl, and 0.1 to 100 μM bicyclomycin (kindly provided by the Ciba-Geigy Pharmaceutical Co., Ltd., Basel, Switzerland), when required. Five minutes later the elongation reaction was carried out in the presence of a concentration of 200 μM (each) ATP, CTP, and GTP, 20 μM UTP, and 5 μCi of [α-^{32}P]UTP (400 Ci mmol^{-1}). The reactions were stopped by the addition of 250 μl of 0.3% (wt/vol) sodium dodecyl sulfate (SDS) and 10 μg of *E. coli* tRNA in 50 mM EDTA. The samples were extracted twice with a 1:1 (vol/vol) mixture of water-satured phenol and chloroform-isoamyl alcohol, and then a precipitate was formed by adding 0.3 M sodium acetate (pH 5) and 2.5 volumes of ethanol. The pellets were collected and dissolved in 80% formamide-dye solution, and the solution was loaded onto a 5% acrylamide denaturing gel. In vitro transcriptions were also performed by omitting the radioactive UTP and carrying out the elongation in the presence of 400 μM (each) ATP, GTP, CTP, and UTP. The unlabelled RNAs were analyzed by S1 nuclease mapping as described above.

RNA gel mobility assay. The standard transcription protocol was performed by incubating, in a volume of 20 μl, 500 ng of linearized pGEM294 DNA in a buffer containing 500 μM (each) ATP, GTP, and CTP, 50 μM UTP, 40 μCi of [α-^{32}P]UTP (400 Ci mmol^{-1}), 10 mM dithiothreitol, 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, and 5 U of purified T7 RNA polymerase (Boehringer Mannheim GmbH) for 60 min at 38°C. Synthesis of large amounts of unlabelled RNA was carried out by omitting [α-^{32}P]UTP and substituting it with 500 μM UTP.

The gel mobility shift assays were performed by mixing purified Rho factor, labelled RNA, competitor RNA (when required), and buffer in a total volume of 20 μl. The final buffer composition was 10 mM Tris acetate (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl, 10 mM dithiothreitol, 50 mM KCl, 5% glycerol, and 0.1 mM ATP (when required). The concentration of Rho was 500 nM, and the concentration of the labelled RNA was 50 nM. Unlabelled competitor RNA was added at an amount 25-fold in excess of the amount of labelled RNA. Yeast RNA (50 μg/ml) was used as a nonspecific competitor RNA when indicated. When required, bicyclomycin was added at a concentration of 100 to 500 μM. The mixture was incubated for 5 min at room temperature before it was loaded onto a 4.5% polyacrylamide gel in 0.5× standard TBE buffer (1× TBE buffer is 0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA). Electrophoresis was carried out at 2 to 4°C.

Direct UV photoaffinity labelling. UV cross-linking was performed essentially as described previously (10). Briefly, triplicate samples of 2 μg of Rho in 50 μl of TKM buffer (50 mM Tris HCl [pH 7.5], 200 mM KCl, 1 mM $MgCl_2$) were UV irradiated in the presence of 0.125 to 25 μM [α-^{32}P]ATP (40 Ci mmol^{-1}) and 0 to 50 μM bicyclomycin. The photoaffinity labelling was performed by placing the samples under a shortwave transilluminator (Globus) at a distance of 8 cm for 15 min at room temperature. The germicidal lamp inside the Globus apparatus screens light at wavelengths below 240 nm. After irradiation the samples were divided into two aliquots. One aliquot was suspended in Laemmli buffer, heated for 2 min at 100°C, and analyzed by electrophoresis on precast 10 to 15% SDS-polyacrylamide gels (PhastGel Gradient 10-15; Pharmacia, Uppsala, Sweden). Quantitative analysis was performed as described above for S1 nuclease mapping. The other aliquot was treated with 2.5 volumes of ice-cold 10% trichloroacetic acid for 10 min at 0°C, filtered on a Millipore HA filter, and dried, and the radioactivity was counted with a TopCount Canberra instrument (Packard, Meriden, Conn.).

Gel filtration techniques. The oligomerization state of the Rho protein was determined under different conditions by gel filtration techniques. The analysis was carried out with a high-pressure liquid chromatography (HPLC) system (Hewlett-Packard HP 1010) connected to a UV detector on a SHODEX KW-803 column (8 by 300 mm; Showa Denco, Tokyo, Japan) equilibrated at room temperature with a buffer containing 20 mM Tris HCl (pH 7.9), 10 mM $MgCl_2$, 500 mM KCl, 0.1 mM dithiothreitol, 10% glycerol, and (when present) 0.2 mM ATP. The mass exclusion limit was 15,000 Da. Samples (50 μl) containing 0.48 mg of Rho per ml, 0.03 mg of poly(C) (average size, of 80 nucleotides) per ml, and, when present, 100 μM bicyclomycin were incubated for 30 min at room temperature and were then applied to the column. The column was previously calibrated by running a molecular weight standard sample for gel filtration (Sigma) containing thyreoglobulin (M_r = 660,000), β-amylase (M_r = 200,000), bovine serum albumin (M_r = 67,000), and bovine carbonic anhydrase (M_r = 29,000). The absorbance was monitored continuously at 280 nm. The fractions were collected by following the absorbance profile and were analyzed by electrophoresis on precast 10 to 15% SDS-polyacrylamide gels (PhastGel Gradient 10-15; Pharmacia).

RESULTS

Effects of bicyclomycin on Rho-dependent transcription termination. To analyze the effects of bicyclomycin on Rho

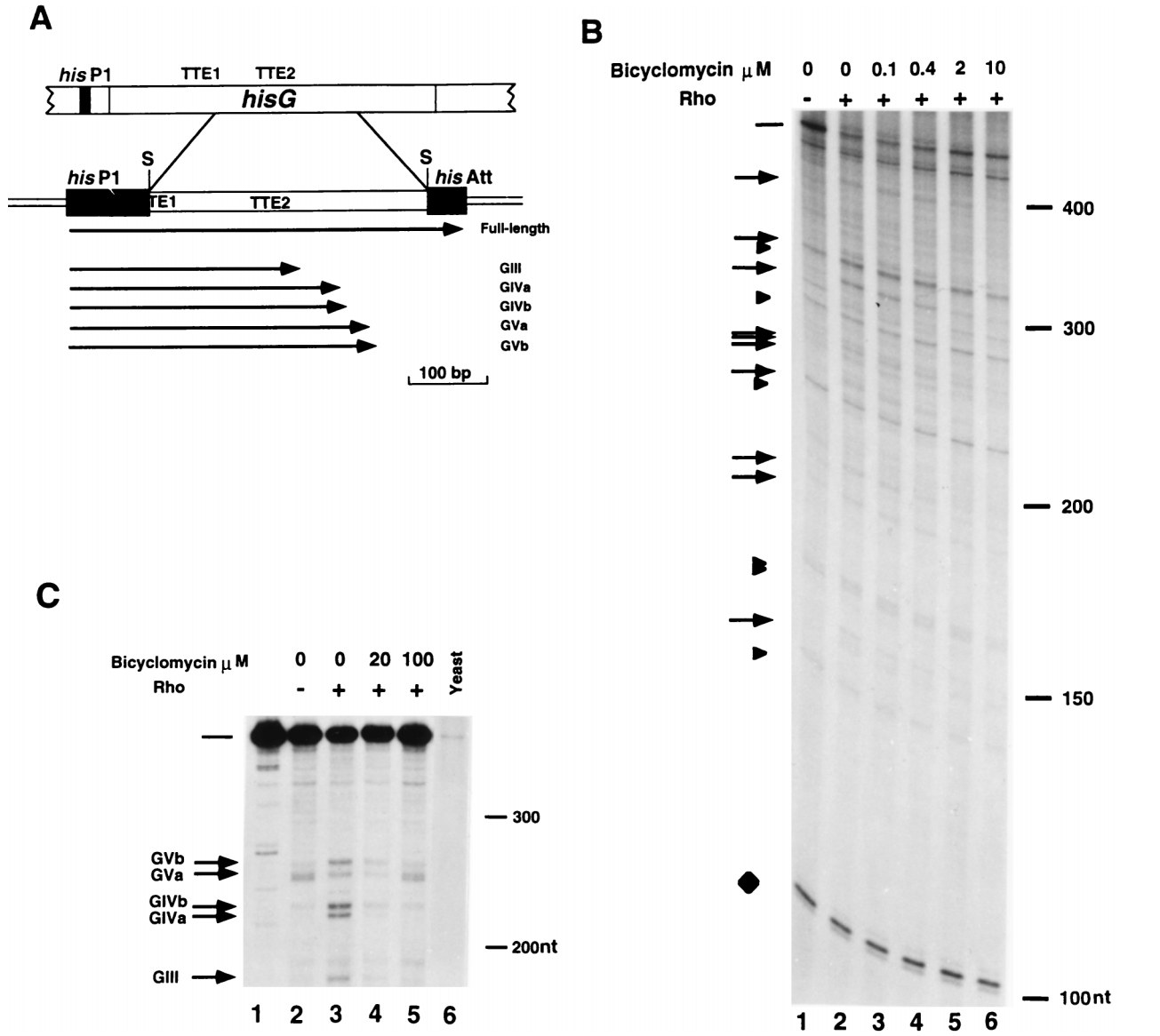

FIG. 1. In vitro Rho-dependent transcription termination in the *hisG* cistron in the presence of bicyclomycin. (A) A genetic map of the promoter-proximal region of the *his* operon of *S. typhimurium* is shown at the top of panel A. The relative positions of the *his* P1 primary promoter, the structural *hisG* gene, and the intracistronic Rho-dependent TTEs (TTE1 and TTE2) are indicated. An enlarged map of the *hisG* region contained in plasmid pHG360 is shown at the bottom of panel A. This plasmid was obtained by cloning a 359-bp *Sau*3AI DNA fragment spanning the TTE2 between the *his* P1 primary promoter and the *his* attenuator (*his* Att) (3). The arrows identify the positions of the 3′ ends and the lengths of the major transcripts produced in vitro. S, *Sau*3AI. (B) Plasmid pHG360 was transcribed in vitro with *E. coli* RNA polymerase and [α-^{32}P]UTP either in the absence (lane 1) or in the presence (lanes 2 to 6) of purified Rho. In lanes 3 to 6 bicyclomycin was added at a concentration of 0.1 to 10 μM. The arrows on the left indicate the positions of the putative Rho-dependent terminated transcripts. Arrowheads correspond to Rho-independent transcripts. The full-length transcript is indicated by a bar. A 104-nucleotide vector-specific Rho-independent transcript (7) is shown as a loading control (diamond). Densitometric values of the full-length transcript in the absence of Rho (lane 1) and in the presence of Rho (lane 2) or Rho plus 100 μM bicyclomycin (lane 6) were determined by normalizing the intensities to the intensity of the 104-nucleotide (nt) transcript. The positions of molecular weight markers (100, 150, 200, and 300 nucleotides) are indicated on the right. (C) S1 nuclease mapping of the transcripts in the *hisG* cistron terminated in vitro. Plasmid pHG360 was transcribed in vitro either in the absence (lane 2) or in the presence (lanes 3 to 5) of purified Rho. Bicyclomycin was added at concentrations of 20 μM (lane 4) and 100 μM (lane 5). The RNAs synthesized in vitro or 20 μg of yeast RNA (lane 6) were hybridized to the 3′-end labelled strand derived from the 359-bp *Sau*3AI fragment complementary to the RNA (lane 1). The hybrids were treated with S1 nuclease and were electrophoresed on a 5% acrylamide denaturing gel. The arrows on the left indicate the more prominent 3′ ends corresponding to Rho-dependent terminated transcripts observed previously (3). The full-length protected transcript is indicated by a bar. The positions of the molecular weight markers (200 and 300 nucleotides) are indicated on the right.

activity we have performed an in vitro transcription assay using as a substrate the intracistronic termination region of *S. typhimurium hisG* (Fig. 1). This region is responsible for the transcriptional polarity of several promoter-proximal *hisG* mutations (8, 9) and is composed of two Rho-dependent transcription termination elements (TTEs), TTE1 and TTE2 (3). The uncoupling of transcription and translation results in partial Rho-dependent termination spread out over multiple weak sites which have been mapped previously (3, 9).

The TTE2 of the *hisG* termination region is present in plasmid pHG360 (Fig. 1A) downstream of the efficient *hisP1* promoter (3). This plasmid was transcribed in vitro with *E. coli* RNA polymerase and [α-^{32}P]UTP either in the absence or in the presence of purified Rho with different amounts of bicy-

TABLE 1. Readthrough transcription in the termination region of the *hisG* cistron in the presence of bicyclomycin[a]

Transcript or site	Level of transcription under the following conditions			
	Without Rho	With Rho	With Rho and bicyclomycin (20 μM)	With Rho and bicyclomycin (100 μM)
RT	10,666 (100.0)	7,523 (69.4)	8,486 (89.1)	10,569 (97.3)
GVb	ND	615 (5.7)	248 (2.6)	ND
GVa	ND	476 (4.4)	204 (2.1)	110 (1.0)
GIVb	ND	883 (8.2)	243 (2.6)	127 (1.2)
GIVa	ND	703 (6.5)	192 (2.0)	ND
GIII	ND	634 (5.8)	157 (1.6)	61 (0.6)
TOT	10,666 (100.0)	10,834 (100.0)	9,530 (100.0)	10,867 (100.0)
T	(0.0)	(30.6)	(10.9)	(2.8)

[a] The levels of radioactivity associated with the relevant bands of the gel shown in Fig. 1C were measured and were compared by densitometry. Values are expressed as densitometric values and as a percentage of the densitometric values (values in parentheses), setting the total radioactivity of each lane (TOT) to 100%. RT, readthrough transcript; T, total Rho-dependent termination efficiency at TTE2 (T = TOT − RT). ND, not detectable.

clomycin (Fig. 1B). Under basal conditions (without Rho and without drug) the expected pattern, consisting of a full-length transcript and shorter molecules corresponding either to prematurely released transcripts or to initiated transcripts from other plasmid promoter sites, was observed (Fig. 1B, lane 1). A densitometric analysis of the gel showed that the addition of Rho to the transcription system reduced the levels of fully elongated transcripts to about 22% and caused the appearance of additional faster-migrating bands corresponding to prematurely terminated transcripts at multiple weak sites (Fig. 1B, lane 2, and data not shown). Bicyclomycin reduced the amount of these faster-migrating bands and increased the amount of the full-length transcript (Fig. 1B, lanes 3 to 6). The decrease in termination efficiency was dependent on the concentration of the drug. At 10 μM bicyclomycin the levels of fully elongated transcripts were about 40% of the levels measured in the absence of Rho and drug.

To determine the position on the nucleotide sequence of the 3′ ends of several Rho-dependent terminated transcripts we analyzed the RNA produced in vitro by S1 nuclease mapping (Fig. 1C). For this procedure the plasmid pHG360 was transcribed in vitro under the conditions mentioned above except that radioactive UTP was omitted and elongation was carried out in the presence of the same concentration of each of the nucleoside triphosphates in order to obtain a larger amount of RNA. Unlabelled RNAs were analyzed by S1 nuclease mapping by using as a probe the 3′-end-labelled strand derived from the 359-bp *Sau*3AI fragment complementary to the RNA (Fig. 1C, lane 1). When transcription was carried out in the absence of Rho the usual pattern (3), consisting almost exclusively of the full-length transcript, was observed (Fig. 1C, lane 2). The addition of Rho to the transcription system caused reduced levels of fully elongated transcripts and the appearance of several faster-migrating bands corresponding to prematurely terminated transcripts (GIII, GIVa, GIVb, and GVb; Fig. 1C, lane 3). Due to the inability of this technique to detect transcripts larger than a certain size, these transcripts represent only a fraction of those generated by the Rho-dependent process (Fig. 1B). As expected, bicyclomycin decreased the Rho-dependent termination efficiency (Fig. 1C, lanes 4 and 5). The inhibitory effect of bicyclomycin was quantified by densitometric analysis (Table 1). The overall efficiency of termination in this experiment was 30%, which was significantly lower than that found in the previous experiment performed with radioactive UTP (Fig. 1B). Because the function of Rho has been shown to depend on the transcriptional elongation rate of RNA polymerase (15), we believe that the higher efficiency found in the previous experiment might be due to the lower UTP concentration which is commonly used to achieve high-specific-activity labelling with [α-^{32}P]UTP. Under these conditions, rates of chain growth are reduced, particularly in uracil-rich regions (4) which are present at the level of TTE2 (3). In this experiment inhibition of Rho-dependent transcription termination was 64% with 20 μM bicyclomycin and maximal with a concentration of 100 μM (Table 1). With 20 μM bicyclomycin, inhibition of transcription termination was more pronounced at the level of the promoter-proximal GIII, GIVa, and GIVb sites than at the level of the more distal GVa and GVb sites (Fig. 1C, lane 4; Table 1).

ATP-independent RNA binding of Rho in the presence of bicyclomycin. The possible interference of the drug on ATP-independent RNA binding of Rho at the primary site has recently been excluded by Magyar et al. (18) on the basis of the results of poly(C)-binding assays. However, it has been reported that some inhibitors of Rho action (e.g., heparin [16]) inhibit binding to natural mRNA but not to poly(C). This may be due to the unusual high affinity of binding of Rho to poly(C). We therefore used a gel mobility shift experiment with a natural mRNA substrate, the TTE2 of *hisG*, to analyze the effects of bicyclomycin on the ATP-independent RNA binding of Rho.

Specific binding of Rho to the RNA was indicated by the appearance of a retarded band (Fig. 2B, lane 2) which disappeared upon competition with 25-fold excess cold probe (Fig. 2B, lane 9) and was not affected by a large excess of yeast RNA, which was added as a nonspecific competitor (Fig. 2B,

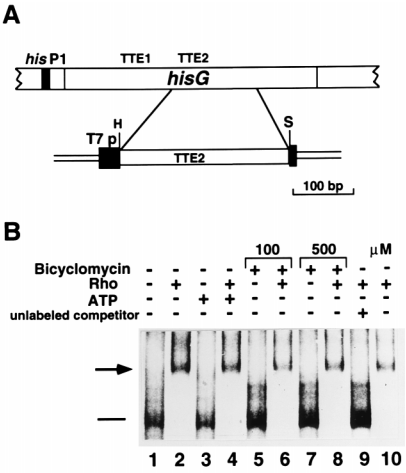

FIG. 2. RNA gel mobility shift assay of ATP-independent binding of Rho to *hisG* RNA in the presence of bicyclomycin. (A) The relative position of the 293-bp *Hin*cII-*Sau*3AI DNA fragment which has been cloned into the polylinker of vector pGEM3Z to obtain plasmid pGEM293 is indicated below the genetic map of the promoter-proximal region of the *his* operon of *S. typhimurium*. The definitions of the symbols and abbreviations are the same as those for the symbols and abbreviations in Fig. 1A. H, *Hin*cII; S, *Sau*3AI; pT7, T7 RNA polymerase promoter. (B) Linearized plasmid pGEM293 was transcribed in vitro with T7 RNA polymerase in the presence of [α-^{32}P]UTP. The RNA synthesized in vitro (bar) was incubated without (lanes 1, 3, 5, and 7) or with (lanes 2, 4, 6, 8, 9, and 10) purified Rho. Bicyclomycin was added at a concentration of 100 μM (lanes 5 and 6) or 500 μM (lanes 7 and 8). Lanes 3 and 4, incubations were carried out in the presence of 0.1 mM ATP. A 25-fold excess of unlabelled probe (lane 9) or 1 μg of yeast RNA (lane 10) was used as specific or nonspecific competitor RNA, respectively. The complexes were analyzed on a 5% polyacrylamide gel. The arrow indicates a specific retarded band corresponding to the Rho-RNA complex.

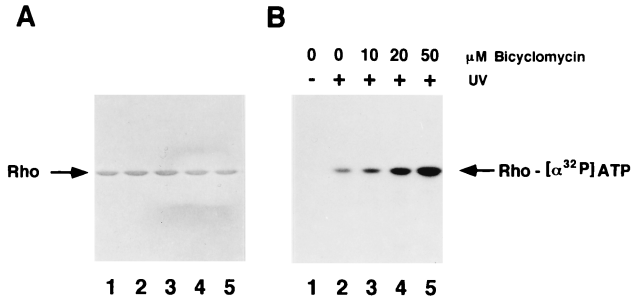

FIG. 3. UV cross-linking of ATP to Rho in the presence of bicyclomycin. Rho samples (2 μg) were not irradiated (lane 1) or were irradiated with UV in the presence of 0.25 μM [α-^{32}P]ATP (40 Ci mmol^{-1}) and 0, 10, 20, and 50 μM bicyclomycin (lanes 2 to 5, respectively). Photoaffinity labelling was analyzed on 10 to 15% SDS-polyacrylamide gels. (A) Coomassie blue staining. (B) Autoradiography.

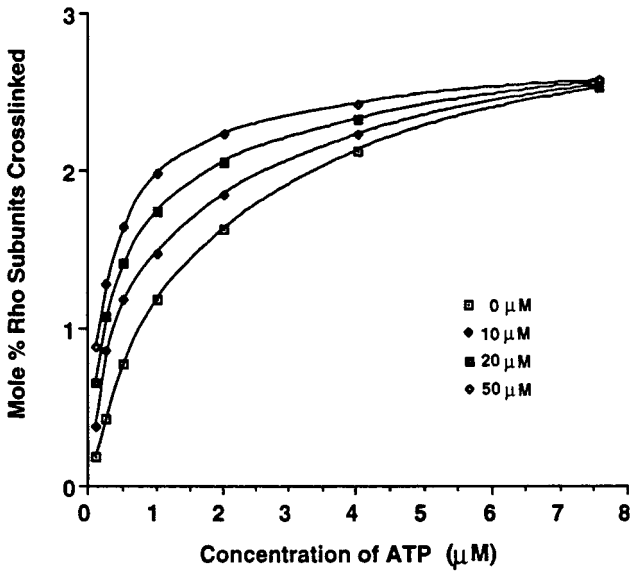

FIG. 4. ATP UV cross-linked to Rho in the presence of several amounts of bicyclomycin as a function of ATP concentration. Rho samples (2 μg) were irradiated with UV in the presence of the indicated concentrations of [α-^{32}P]ATP (40 Ci mmol^{-1}) and bicyclomycin. After irradiation the samples were treated with trichloroacetic acid, filtered on a Millipore HA filter, and dried, and the radioactivity was counted. Values are means for triplicate samples. The mole percentage of the Rho subunits that cross-linked is equal to the number of moles of ATP precipitated per mole of subunit × 100.

lane 10). The binding detected under these conditions corresponds to the interaction of Rho with the primary site on the RNA. Consistently, it was not modified by the presence of ATP (Fig. 2B, lane 4). The amount of the retarded Rho-RNA complex was not affected by preincubation of Rho with inhibitory concentrations of bicyclomycin (Fig. 2B, lanes 6 and 8). This finding indicated that the drug does not interfere with the ATP-independent RNA-binding activity of Rho.

ATP binding of Rho in the presence of bicyclomycin. The evidence that bicyclomycin does not block the RNA-binding activity of Rho presented above suggested that the drug might interfere with a process more directly linked to the Rho ATPase function. Therefore, we measured the ATP-binding activity of Rho in the presence of bicyclomycin by photochemical cross-linking. This method is commonly used to measure ATP binding to wild-type (10) or mutated (21) Rho. The Rho-ATP adducts were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and were analyzed by autoradiography (Fig. 3). According to a previous report (10) the labelling was time dependent and saturable by ATP. Under our conditions Rho was saturated at 7.5 μM ATP by irradiating the samples for 15 min at room temperature (data not shown). We used an ATP concentration of 0.25 μM to investigate the effect of bicyclomycin. The results showed the ATP labelling of Rho after UV irradiation (Fig. 3B, lane 2). The signal on the autoradiograph coincides with the single band visible after Coomassie staining of the gel (Fig. 3A). The presence of bicyclomycin increases the level of binding of ATP to Rho (lanes 3 to 5). Quantitative analysis was performed by densitometry (Table 2).

In a second experiment we determined the amount of UV-cross-linked ATP in the presence of several different amounts of bicyclomycin as a function of the ATP concentration. For this purpose, after irradiation the samples were divided into two aliquots. One aliquot was treated with trichloroacetic acid, filtered on a Millipore HA filter, and dried, and the radioactivity was counted. The result of this experiment is presented in Fig. 4. The other aliquot was analyzed by SDS-PAGE, and quantitative analysis was performed by directly counting the radioactivity bands with a PhosphoImager. Results comparable to those obtained in the first experiment were obtained by this second method (data not shown). Scatchard analysis of the binding data indicated that the apparent equilibrium dissociation constant ($K_{D,app}$) was 0.970 μM in the absence of bicyclomycin, which is in good agreement with the value reported by Dolan et al. (10). The presence of 50 μM bicyclomycin decreased the $K_{D,app}$ value to 0.405 μM.

TABLE 2. Binding of ATP to Rho in the presence of bicyclomycin[a]

Lanes in Fig. 3	Sample	Density values
1	Rho not irradiated	0
2	Rho	294
3	Rho + bicyclomycin (10 μM)	797
4	Rho + biocyclomycin (20 μM)	903
5	Rho + bicyclomycin (50 μM)	1,111

[a] Samples containing 2 μg of Rho, 0.25 μM [α-^{32}P]ATP (40 Ci mmol^{-1}), and, where indicated, bicyclomycin were UV irradiated as described in the Materials and Methods section. Aliquots were boiled for 2 min in Laemmli buffer and were subjected to SDS-PAGE (see the legend Fig. 3). The radioactivity associated with the Rho band of each sample was measured and compared by densitometry.

Effects of bicyclomycin on oligomerization state of Rho. The oligomerization state of Rho under various conditions can be estimated by comparing its mobility upon gel filtration in a column with the mobilities of proteins with known apparent molecular weights run on the same column (13). It has been reported that in vitro state of oligomerization of Rho is influenced by the ionic strength of the solution and by the presence of ATP and poly(C) (13). A high ionic strength favors the dissociation of Rho into monomers. In contrast, the presence of ATP appears to shift the equilibrium in favor of oligomerization (tetramers). Binding to poly(C) results in the stabilization of Rho in the hexameric form under ionic conditions as well, which favors the dissociation of the subunits. Under these conditions, stabilization requires the presence of ATP.

Since bicyclomycin appeared to affect the binding of ATP to Rho, gel filtration experiments were performed to analyze the effects of the drug on the formation of Rho hexamers at high ionic strength and in the presence or absence of both ATP and poly(C) (Fig. 5).

In a preliminary experiment, purified Rho protein was ap-

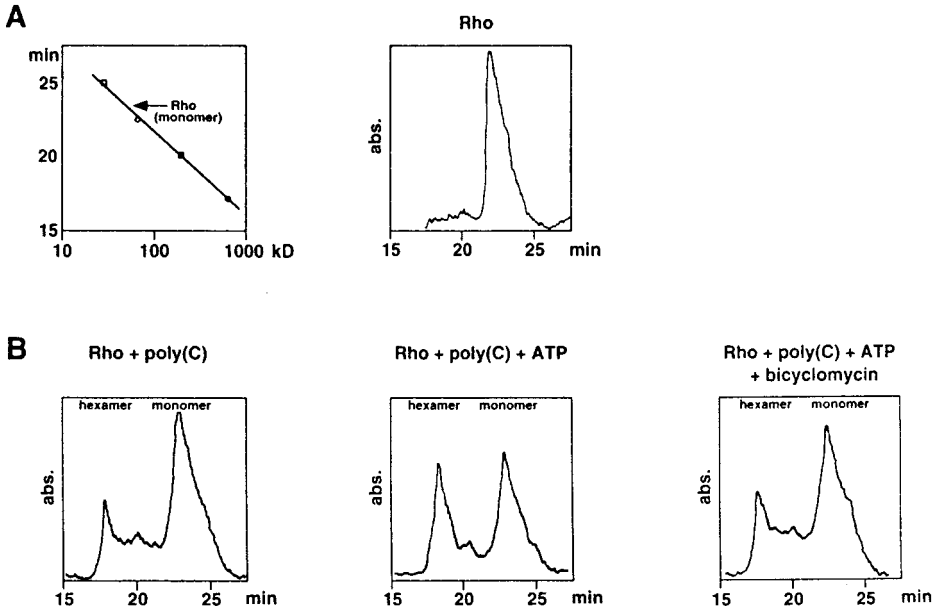

FIG. 5. Hexamer formation of Rho polypeptides in the presence of bicyclomycin. (A) Samples containing 0.48 mg of Rho per ml were applied to a SHODEX KW-803 column in the absence of both ATP and poly(C). The relative mobility of the Rho monomer (right) is related to the mobilities of reference proteins (left). abs, absorbance at 280 nm; min, minutes of elution. (B) The relative abundance of the Rho monomer and the Rho hexamer is indicated within each chromatogram when Rho samples (0.48 mg/ml) were applied to the column in the presence of poly(C) (0.03 mg/ml) and, when indicated, in presence of ATP (0.2 mM) and bicyclomycin (100 μM).

plied at a concentration of 0.48 mg/ml to a previously calibrated SHODEX KW-803 column mounted on an HP 1010 high-pressure liquid chromatograph and was eluted at high ionic strength (500 mM KCl). The elution profile was followed by continuously monitoring the A_{280}. Peak fractions were also analyzed by SDS-PAGE (data not shown). In the absence of both poly(C) and ATP, the peak in the elution profile corresponded to the position expected for Rho monomers (Fig. 5A). When poly(C) with an average chain length of 80 nucleotides, which is large enough to saturate the single large RNA secondary site on Rho but small enough to bind to only one Rho molecule (13), was added at a concentration of 0.03 mg/ml at a 1:1 molar ratio with respect to the amount of Rho hexamers, the ratio between the fraction of the molecules in the hexameric form and those in the monomeric form was about 1:4 (Fig. 5B). The deduced molecular mass of hexameric Rho-poly(C) complex was about 345,000 Da, which is the value expected for a hexamer of a polypeptide of 48,000 Da, assuming that one poly(C) molecule is bound per Rho hexamer. A minor peak of protein eluting at about 20 min was also detectable and corresponded to a dimer. As expected, the ratio between the fraction of the molecules in the hexameric form and those in the monomeric form was further increased (to about 1:1) by the presence of a large molar excess of ATP (0.2 mM). The addition of bicyclomycin to the Rho and poly(C) mixture in the presence of 0.2 mM ATP resulted in a decrease of this ratio. Quantitative analysis of the major peak fractions in the chromatogram showed a ratio of 1:3 in the presence of 100 μM bicyclomycin.

DISCUSSION

In the present study we have used the intracistronic termination region of *hisG* as an experimental system to study the mechanism of inhibition of the Rho-dependent process by bicyclomycin. This region is composed of two sequential TTEs responsible for termination at multiple termination sites (TSs) (3, 9). Intracistronic regions with similar features are widespread throughout the genome and are responsible for the phenomenon of transcriptional polarity. The physiological significance of this Rho-dependent process is the prevention of the synthesis of unused transcripts during conditions of physiological stress (1, 2, 19, 28, 30, 32).

We show that bicyclomycin prevents transcription termination in vitro at the TTE2 of the intracistronic termination region of *hisG*. The amount of bicyclomycin that gives 50% inhibition of transcription termination at the level of the entire TTE2 (I_{50}) is 15 to 20 μM. This value is about threefold higher than that observed with the *trpt'* system (18) but more than threefold lower than that measured when poly(C) is used as a substrate (24). These variations might be attributed to differences in both the nature of the substrates and the experimental procedures.

Significantly, the I_{50}s at the level of the single TSs of the TTE2 were different. Bicyclomycin inhibition was more pronounced at the level of the promoter-proximal GIII, GIVa, and GIVb than at the level of the more distal GVa and GVb TSs (Table 1). This phenomenon is consistent with the idea that bicyclomycin inhibition of the Rho-dependent process is due to its ability to affect the Rho secondary RNA-binding site and to slow the rate of tracking toward the RNA polymerase. This phenomenon has the same significance as the phenomenon observed by Magyar et al. (18) at the level of the *trpt'*, where a new set of more distally terminated transcripts appeared upon the addition of bicyclomycin at concentrations close to the observed I_{50}.

In an attempt to elucidate the molecular mechanism of bicyclomycin inhibition of Rho activity, we have investigated the possible interference of the drug with the ATP-independent RNA-binding activity of Rho with a natural substrate, the Rho loading site of the transcription termination region of *hisG*. The RNA gel mobility shift experiments (Fig. 2) enabled us to detect the ATP-independent formation of a Rho-RNA com-

plex. The results of this experiment excludes an effect of bicyclomycin on the ATP-independent RNA binding of Rho, suggesting a direct interference of the drug with the inherent ATPase activity of Rho.

We therefore measured the ATP-binding ability of Rho in the presence of bicyclomycin by photochemical cross-linking experiments (Fig. 3). Because of the lack of an RNA substrate in these assays, ATP was not converted to ADP plus P_i. This allowed us to measure the affinity of Rho for ATP (10). We found that bicyclomycin increases the amount of ATP cross-linked to Rho (Fig. 4). The effect of the drug on ATP binding was specific because at higher ATP concentrations the amount of ATP bound reaches a plateau with all drug concentrations tested. This ruled out the possibility that bicyclomycin might enhance a somewhat nonspecific binding of negatively charged ATP to the highly basic Rho polypeptide. Scatchard analysis of the binding data indicates that under these experimental conditions, the $K_{D,app}$ is reduced by about one-half in the presence of 50 μM bicyclomycin.

However, the possibility that bicyclomycin could be increasing the efficiency of cross-linking still exists. This effect might be due to a presumed ability of the drug to induce in the protein conformational changes that optimize the interaction of the ATP adenine ring and/or the phosphoryl groups with critical cross-linking amino acids on the Rho polypeptide. Alternatively, bicyclomycin might increase the reactivities of these residues within the hydrophobic ATP-binding pocket. These considerations would support the idea that bicyclomycin inhibition of Rho-dependent processes involves the binding of the antibiotic at or near the ATP-binding domain.

It has been described previously that bicyclomycin acts through a simple noncompetitive mechanism of inhibition with respect to ATP (24). As a consequence, the drug would not change the K_m for ATP in the Rho-ATPase reaction (11 μM). Here we found that bicyclomycin indeed does not compete for the binding of ATP to Rho. On the contrary, it apparently increases the ATP-binding ability of Rho by reducing the $K_{D,app}$. This apparent discrepancy might be due to measurement of a binding parameter ($K_{D,app}$) and not a kinetic parameter (K_m). Moreover, in the case of the Rho-ATPase reaction, the K_m (11 μM) is substantially different from the $K_{D,app}$ for ATP, that is, close to 1 μM in the photochemical cross-linking experiment (10) and five times lower in the direct-binding studies (36).

Enzymatic kinetic studies have revealed that hydrolysis of ATP is coupled with the interaction of Rho with RNA segments at a low-affinity (secondary) site (28, 29, 35). As a consequence of the interference on Rho-ATP binding and hydrolysis, bicyclomycin might affect the secondary RNA-binding (tracking) site on Rho. Strong evidence for such a mechanism has been proposed in a recent study which appeared while the present work was in progress (18). The experimental evidence is based on in vitro transcription termination assays and poly(dc)-poly$(C)_{10}$-stimulated ATPase assays. In those studies bicyclomycin inhibition followed a mixed inhibition model with respect to poly$(C)_{10}$.

The results of the cross-linking experiments presented here suggest that bicyclomycin might alter the interaction of the ATP adenine ring and/or the phosphoryl groups with critical Rho residues within the hydrophobic ATP-binding pocket, resulting in an increased affinity for the nucleotide. Bicyclomycin might therefore disturb the correct positioning of ATP within the pocket which in turn is required for adequate exposure of the phosphoryl groups to amino acid residues which are directly involved in catalysis. Such perturbation of ATP binding to Rho might explain the proposal (18) that bicyclomycin affects the RNA tracking site and slows its progression toward bound RNA polymerase.

Our hypothesis is supported by the nature of three missense mutations in the *rho* gene, each of which confers bicyclomycin resistance. Two of these mutations, SA266 and MK219, were found in the central 270-amino-acid region constituting the putative ATP-binding domain (39). More interestingly, the third mutation, GS337, occurred in the carboxy-terminal half of the protein; it has been proposed that this mutation plays a pivotal role in functionally coupling the RNA- and ATP-binding domains (21). Mutants with mutations in this region often have multiple phenotypes (21). For instance, the EK392 mutation reduces to half the $K_{D,app}$ for ATP and causes a tenfold increase in the K_m for poly(C) in the Rho-ATPase reaction. The two pieces of evidence that bicyclomycin apparently modifies the $K_{D,app}$ for ATP and the K_m for poly$(C)_{10}$ at the same time are therefore consistent with this model.

It has been extensively documented that the hexamer form of Rho is the functionally active form of the Rho molecule (13). Nevertheless, the ability of Rho to dissociate into monomers may be important in the catalytic cycle of ATP hydrolysis. In a recent model of quaternary structure, hexameric Rho has a ring-shape structure in which six globular subunits are arranged around a hollow core. The six subunits in the hexamer would be oriented with all the primary RNA-binding domains on one face of the ring and with the parts of the subunits containing the ATP-binding domain in the inner hole of the ring. The finding that many of the mutations that affect interactions with RNA are clustered in the segment that extends into the hole has suggested that the hole also contains the secondary RNA-binding site. According to this view, after binding of the RNA to the primary binding site on the Rho surface, a dissociation and reassociation of one or more subunits would allow the interaction of the RNA with the secondary site in the hole (31). We have found that bicyclomycin reduces the proportion of Rho molecules in the hexameric form in the presence of both poly(C) and ATP. ATP binding, which occurs with a stoichiometry of three ATP sites per Rho hexamer (36), has been found to stabilize the hexameric form of Rho in the presence of poly(C) (13). An explanation for these effects is that a single ATP-binding site is shared between adjacent subunits (13). Alternatively, ATP binding might induce conformational changes that would result in a stronger interaction between subunits (13) and in a negative cooperativity that prevents nucleotide binding to subunits adjacent to occupied active sites (11, 17). Therefore, we believe that the effects of bicyclomycin on the oligomerization state of Rho might be due to its ability to disturb the correct positioning of ATP within the ATP-binding pocket. Alternatively, on the basis of the model mentioned above (31), a primary defect in hexamer formation might interfere with ATP-binding and with the binding of RNA at the level of the secondary (tracking) site in the hole of the ring-shape hexameric structure.

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