Evidence for the Location of Bicyclomycin Binding to the 
Escherichia coli Transcription Termination Factor Rho*

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The commercial antibiotic bicyclomycin (Bcm) has been shown to target the essential transcription termination factor Rho in Escherichia coli. Little is known about the Bcm binding domain in Rho. A recent structure-activity relationship study led us to evaluate the reductive amination probe, 5α-(3-formylanilino)dihydrobicyclomycin (FD-Bcm). Biochemical studies showed that FD-Bcm possessed inhibitory activities comparable to Bcm in Rho-dependent ATPase and transcription termination assays. Incubation of Rho with FD-Bcm, ATP, and poly(C) followed by NaBH4 reduction and dialysis led to an appreciable loss of ATPase activity. Inclusion of Bcm with FD-Bcm in the reductive amination reaction protected Rho, indicating that Bcm and FD-Bcm competed for the same binding site in Rho. Incubation of Rho with FD-Bcm and poly(C) followed by NaBH4 reduction provided a sample with residual ATPase activity (12%). Mass spectrometric analysis identified the presence of two proteins in an approximate 1:2:1 ratio, whose masses corresponded to wild-type Rho (47,010 Da) and lysine-modified Rho (47,417 Da), respectively. Trypsin digestion of the Rho sample followed by high performance liquid chromatography separation and tandem mass spectrometry analysis identified the site of modification as Lys181 within the combined trypic fragment, Gly-Leu-Ile-Val-Ala-Pro-Pro-Lys-Ala-Gly-Lys (residues 174–184). Similar analysis of a lesser modified sample (following incubation with inclusion of ATP) showed that addition had again occurred at Lys181. These findings provide the first structural information concerning the site of Bcm binding in Rho.

Bicyclomycin (Bcm)† is a commercial antibiotic of novel structure (1–8). The primary site of action in Escherichia coli is unique and has been identified as the transcription termination factor, Rho (9). Rho is composed of six identical 47-kDa proteins of 419 amino acids (10) and exists in a planar, hexagonal (11–13) arrangement of proposed C6 (14) or D3 (15) geometry. Rho-dependent transcription termination sites occur at the ends of transcription units, at regulatory points before or between genes, and within genes (16). Transcription termination begins with the recognition and binding of Rho to discrete regions (rut sites) within the newly synthesized RNA chains (17). Binding is governed by a general requirement for a cytosine-rich sequence, and it extends across all six subunits of the functional protein, encompassing approximately 80 nucleotides (17–20). Rho then extends its interaction toward the 3′ end of the RNA polymerase elongation complex in a process coupled to ATP hydrolysis. The Rho-mediated release of RNA from the transcription bubble results from a destabilization of the complex, believed to be caused, in part, by the ATPase-dependent 5′ → 3′ RNA-DNA helicase action of the protein (21).

Details of the Bcm-Rho interaction have emerged in recent investigations (9, 22–24). Chemical studies have shown that nucleophilic amino acids readily react with Bcm, consistent with several early mechanistic proposals for the mode of action of this antibiotic (25–28). Kinetic studies, however, demonstrated that Bcm inhibits Rho by a reversible, noncompetitive pathway with respect to ATP under the assay conditions employed (22). Furthermore, analogues of Bcm such as dihydrobicyclomycin (29), which lack the exomethylene moiety and cannot undergo covalent addition to Rho, nevertheless inhibit Rho-mediated activities.

Efforts to obtain further information on the mode of action of Bcm have been hindered by the paucity of structural data on the Bcm-Rho interaction. Attempts to achieve selective, irreversible modification of Rho by Bcm have been unsuccessful. Incubation of Rho with increasing concentrations of highly purified Bcm led to a steady decrease in Rho-dependent poly(C)-stimulated ATPase activity after dialysis (22). The irreversible inactivation percentage did not plateau at concentrations above the levels required to completely inhibit ATPase activity and was proportional to the Bcm concentration employed. Extended incubation of Rho with high molar excesses of Bcm gave rise to a heterogeneous mixture with multiple Bcm
adducts (22). Detailed structural studies provided no evidence of site-specific modification. 2

The use of analogues acting as irreversible inactivators has proven to be a useful approach for the identification of substrate-binding sites in enzymes (30). A recent structure-activity relationship study of Bcm (31–33) has identified 5a-(3-formylanilino)dihydrobicyclomycin (FD-Bcm) as a potential reductive amination probe that warranted further study (34). FD-Bcm has Rho inhibitory properties comparable with Bcm. The I50 value for FD-Bcm was 70 μM in the poly(C)-dependent ATPase assay and ~10 μM in the Rho-dependent transcription termination assay. The corresponding values for Bcm were 60 and 5 μM, respectively. Adding NaN3H to a preincubated solution containing Rho and FD-Bcm (1 mM) led to the efficient ATPase inactivation (74%) of the protein, after removal of unbound FD-Bcm. Subsequent mass spectrometric analyses of the FD-Bcm-Rho adduct showed ~40% addition and indicated a molecular weight for the adduct of approximately 47,410, consistent with a modification of a Rho lysine residue by FD-Bcm (34).

In this paper, we report the binding properties of FD-Bcm, demonstrate that FD-Bcm and Bcm compete for the same binding site in Rho, and show that FD-Bcm functions as a site-specific reductive amination probe identifying a key amino acid residue within the bicyclomycin binding pocket.

**Structure 2. Structure of 5a-(3-formylanilino)dihydrobicyclomycin (FD-Bcm).**

**EXPERIMENTAL PROCEDURES**

**Materials and Enzymes—**Bicyclomycin (Bcm) was purified by three successive silica gel chromatographies using 20% methanol/chloroform as the eluent, as described (22). The synthesis of 5a-(3-formylanilino)dihydrobicyclomycin, FD-Bcm was described previously (22). 3 Rho protein was isolated from E. coli AR120 containing the overexpressing plasmid p39-AS (35) following previously published protocols (35). Rho purity was determined by SDS-polyacrylamide gel electrophoresis, and concentrations were determined according to the Lowry protein determination (36). [γ-32P]ATP was purchased from NEN Life Science Products. HPLC grade water and acetonitrile were purchased from Rathburn. All other chemicals were reagent grade.

**Determination of Poly(C)-dependent Rho ATPase Activity—**The ATPase activity assays were carried out in 100-μl reactions containing 40 mM Tris-HCl, pH 7.9, 50 mM KCl, 12 mM MgCl2, 0.1 mM EDTA, and 0.1 mM DTT, Rho (10 μg, 1 μM), ATP (1 mM), and 0.5 μM of (γ-32P)ATP. The reaction mixture was dialyzed at 25 °C (4 h). An aqueous solution (20 μl) of NaN3H (600 μM) was added, and the reaction was allowed to stand at 25 °C (20 min). The reaction mixture was dialyzed (5 °C, 20 h) against 100 mM NaCl, 10 mM Tris-HCl, pH 7.6, 5% glycerol, 0.1 mM EDTA, and 0.1 mM DTT. The percentage inactivation of Rho was measured by determining the initial velocity of ATPase activity using the procedure described above and adding poly(C)(40 nM), ATP (250 μM), and 0.5 μM of (γ-32P)ATP. Following incubation, the sample was dialyzed against water overnight with three changes of solvent using Spectra/ Por cellulose ester dialysis tubes (500-μl volume; 10,000 molecular weight cut-off; Pierce and Warriner, Chester, UK). Alternatively, HPLC grade water and acetonitrile were used to isolate the protein product. HPLC used a Fluor Instruments Rheos 4000 reciprocating piston pump (Jones Chromatography, Mid Glamorgan, UK) and a polymeric PLRP-S column (50 × 0.5 mm, 40-μm mesh size; Michrom BioResources, Pleasanton, CA) operated at a flow rate of 50 μl/min. The column was eluted with water (incorporating 0.1% formic acid) for 10 min, after which proteins were eluted during a rapid (1 min) gradient to acetonitrile/water (8:2, with 0.1% formic acid) for 10 min, after which proteins were eluted during a rapid (1 min) gradient to acetonitrile/water (8:2, with 0.1% formic acid); the final composition was held for 20 min prior to re-establishment of starting conditions. The injection volume was 20 μl. Formic acid (0.5% by volume of incubation) was added to each sample prior to injection, to improve retention on the liquid chromatography column. Collected protein fractions were analyzed by electrospray MS.

**Detection of the FD-Bcm-modified Rho Amino Acid Residue—**The reductive amination of Rho by FD-Bcm (1 mM) was conducted using the procedure described above in the presence of poly(C)(40 nM) and with or without ATP (1 mM). Following incubation, the sample was dialyzed against ATPase buffer and was subjected to either partial or complete tryptic hydrolysis. For partial hydrolysis, the product of reductive amination was labeled with 15 μl with respect to poly(C) and 15 μl with respect to ATP. Freshly prepared trypsin solution in ATPase buffer was added to give a final concentration of 0.33 μM. The solution was maintained at 37 °C for 30 min prior to quenching by the addition of 0.5% by volume of formic acid. Before MS analysis, the product was desalted using the fast gradient HPLC method described above.

Complete tryptic hydrolysis was achieved by adding trypsin to give a ratio of substrate to enzyme of 50:1 (w/w). The solution was maintained at 37 °C for 18 h, after which the hydrolysate was quenched by the addition of formic acid (0.5% by volume). Fractionation of the hydrolysate was achieved using an Analyst gradient HPLC system (Perkin-Elmer) fitted with a rhodneye (Catot) 7125 injector with a 100-μl loop. Separations were performed on a Partisil C18 column (4.6 × 150 mm; Phase Separations, Deeside, UK) eluted at 1 ml/min. The aqueous phase (solvent A) contained 0.1% trifluoroacetic acid, and the organic phase (solvent B) was acetonitrile containing 0.07% trifluoroacetic acid. A linear gradient was applied from 0 to 100% solvent B over 80 min. The injection volume was 100 μl, and the chromatogram was monitored by UV absorbance at 217 nm using an Applied Biosystems (Foster City, CA) 783A detector. HPLC fractions were collected in 1.5-ml polypropylene tubes and dried under vacuum.

**Electrospray MS—**Samples were performed using a Micromass Quattro (Manchester, UK) upgraded to Quattro II specifications. The capillary potential was held between 3 and 4 kV, and the cone was at 20–30 V. All potentials are reported relative to the skimmer lens. Conventional mass spectra were recorded at a resolution set to give a peak width at half-height of 0.4 m/z units for a monoisotopic peak of a singly charged ion. Protein spectra were accumulated for 5 min under
Location of Bicyclomycin Binding to Rho

The reaction was conducted using a solution (0.2 ml) containing reaction buffer, Rho (10 μg, 1 μM), poly(C) (40 nM), and ATP (1 mM). The presence of additional substrates is indicated in the table for each experiment. The solution was incubated at 25 °C (4 h), treated (where indicated: /, treated; x, not treated) with NaBH₄ (60 μM), and then dialyzed. The experiments were done in duplicate; differences between duplicates were 1–6%.

| Entry | FD-Bcm | Bcm | 3-Carboxybenzaldehyde | NaBH₄ | ATPase activity % |
|-------|--------|-----|-----------------------|------|-------------------|
| 1     | 0      | 0   | 0                     | x    | 100               |
| 2     | 0      | 0   | 1000                  |      | 84.4              |
| 3     | 0      | 0   | 0                     | 100  | 83.8              |
| 4     | 100    | 0   | 0                     |      | 37.3              |
| 5     | 100    | 100 | 0                     |      | 51.5              |
| 6     | 100    | 200 | 0                     |      | 55.4              |
| 7     | 100    | 400 | 0                     |      | 66.4              |
| 8     | 100    | 1000| 0                     |      | 70.2              |
| 9     | 0      | 1000| 0                     |      | 75.3              |

RESULTS

Kinetics of FD-Bcm Binding—The kinetics of FD-Bcm inhibition of the poly(C)-dependent ATPase activity was determined from the rates of ATP hydrolysis at various ATP concentrations (9.1, 11.1, 14.3, 20.0, 33.3, and 100 μM) and at FD-Bcm concentrations from 0 to 60 μM. Fig. 1A is a plot of 1/(rate of ATPase activity) versus 1/[ATP] at various concentrations of FD-Bcm. Our findings indicated that FD-Bcm inhibited poly(C)-dependent ATP hydrolysis by a noncompetitive, reversible pathway with respect to ATP. A similar result was observed for Bcm (22). The Kin value for ATP in our study was 28.0 ± 0.9 μM, which was similar to values previously reported in studies with Bcm (11.0 ± 0.5 μM) (37, 39, 40). The intercepts and slopes of Fig. 1A plotted against the concentration of FD-Bcm showed a linear response (Fig. 1B). The Kin value for FD-Bcm inhibition of Rho ATP hydrolysis was 27 μM, which was comparable with the value found for Bcm (20 μM) (22). The similarity of the Bcm and the FD-Bcm kinetic studies demonstrated that FD-Bcm is an effective inhibitor of Rho-dependent ATP hydrolysis and provided support that this probe binds to the same site as Bcm.

Use of Reductive Amination Probe FD-Bcm in the Presence of Bcm—Prior to the use of FD-Bcm as a site-selective reductive amination reagent, it is necessary to demonstrate that permanent inactivation of Rho-ATPase activity by FD-Bcm is a consequence of covalent modification at the Bcm binding domain in Rho. Accordingly, we performed the reductive amination experiments of Rho with FD-Bcm in the presence and absence of Bcm. We used 100 μM solutions of FD-Bcm that contained saturating amounts of poly(C)(40 nM) and ATP (1 mM) and varied the Bcm concentration from 0 to 1 mM. In a control experiment, FD-Bcm was replaced by 3-carboxybenzaldehyde to determine the extent of Rho inactivation produced by a nonspecific reductive amination reagent.

Low levels of inactivation (~16%) of Rho-ATPase were observed in the control experiments (Table I, entries 2 and 3). Inclusion of only Bcm (1 mM) in the reaction led to ~25% Rho inactivation (Table I, entry 9). This value was near the level of Rho inactivation found in experiments using NaBH₄ alone (Table I, entry 2) and using the control substrate, 3-carboxybenzaldehyde, and NaBH₄ (Table I, entry 3). We have attributed this loss of activity to nonspecific inactivation of Rho that occurred during the incubation and dialysis phases of the experiments. Addition of FD-Bcm (100 μM) in place of Bcm led to
significant losses of ATPase activity (~63%) (Table I, entry 4). Decreased levels of Rho inactivation (~48%) were observed when equal amounts (100 μM) of FD-Bcm and Bcm were used in the reaction (Table I, entry 5). The protective effect provided by Bcm increased with increasing concentrations of Bcm (Table I, entries 5–8). We observed near full recovery of Rho-ATPase activity when a high concentration of Bcm (1 mM) was included in the reaction with FD-Bcm (100 μM) (Table I, entry 8). These findings indicated that FD-Bcm competed with Bcm for the Bcm binding domain in Rho.

Efficiency of Reductive Amination Probe FD-Bcm, the Effect of Cofactors—Investigations have demonstrated that poly(C) promotes active hexameric Rho formation and that ATP induces Rho conformational changes (41, 42). Accordingly, we

Fig. 2. Mass spectrometric analysis of the product of reductive amination of Rho with FD-Bcm. The spectra were obtained by maximum entropy processing of mass/charge ratio data. The reaction mixtures contained the following substrates: A, none; B, FD-Bcm (1 mM); C, FD-Bcm (1 mM) and ATP (1 mM); D, FD-Bcm (1 mM) and poly(C) (40 nM); E, FD-Bcm (1 mM), poly(C) (40 nM), and ATP (1 mM).
determined the effect of co-incubation of these two ligands on the level of inactivation of Rho-ATPase activity by FD-Bcm. The data (not shown) are briefly summarized as follows. First, incubation of FD-Bcm with Rho in the presence of ATP alone gave approximately 50% permanent inactivation after NaBH₄ treatment, when compared with controls. Second, inclusion of poly(C) alone resulted in Rho inactivation levels that reached ~93%. Third, ATP in the presence of poly(C) provided significant protection against permanent inactivation of Rho by FD-Bcm.

Detection of an Irreversible FD-Bcm-Rho Adduct—We have shown that NaBH₄ treatment of a solution containing Rho and FD-Bcm leads to the irreversible loss of ATPase activity and that the extent of Rho-ATPase inactivation depends upon the presence or absence of ATP and poly(C). Electrospray MS has been used to assess the correlation of Rho-ATPase activity with the stoichiometry of covalent modification.

Reductive amination of Rho by FD-Bcm was performed in the presence or absence of cofactors. The concentration of FD-Bcm was either 1 or 5 mM. The electrospray MS data for the reaction products included prominent signals for two different proteins corresponding to approximately 47,010 and 47,417 Da (Fig. 2). We have assigned the two protein signals to Rho and the FD-Bcm-Rho-reduced amine, since reductive amination of Rho by FD-Bcm leads to a predicted increase in molecular weight of 407. A signal (~47,817 Da) corresponding to a disubstituted FD-Bcm-Rho adduct was additionally observed following incubations when high levels (5 mM) of FD-Bcm were employed.

Table II lists the relative proportions of wild-type Rho and mono- and disubstituted FD-Bcm adducts for a series of reductive amination reactions, together with the percentage ATPase activity of the Rho sample prior to MS analyses. For the Rho solution treated with FD-Bcm (1 mM) in the absence of poly(C) and ATP, we observed a 2.4:1 ratio of Rho to FD-Bcm-modified Rho. The Rho-dependent ATPase activity of this solution prior to MS analysis was 28% of that of untreated Rho. Inclusion of ATP (1 mM) in the reductive amination experiment led to slightly lower levels (3.5:1 ratio) of the FD-Bcm-modified Rho and higher levels of ATPase activity (40%). Correspondingly, use of only poly(C) (40 nM) in the reaction led to enhanced levels of adduction (1.2:1 ratio) and decreased ATPase activity (12%). Finally, decreased amounts of the FD-Bcm-modified Rho adduct (4.9:1 ratio) and enhanced levels of ATPase activity (42%) were observed in the presence of both poly(C) (40 nM) and ATP (1 mM). The levels of FD-Bcm adduction were inversely proportional to the ATPase activity. A similar trend was observed when 5 mM FD-Bcm was employed in the reaction, with a single exception (Table II, entry 8). The data reinforce our conclusion.

FIG. 2—continued
that the inactivation of Rho-ATPase activity by FD-Bcm was associated with the covalent modification of Rho by FD-Bcm.

Detection of the FD-Bcm-modified Rho Amino Acid Residue—The detection of FD-Bcm-modified Rho by conventional MS does not permit us to conclude that adduction proceeded at a specific site in Rho. Information concerning the site selectivity of reductive amination probe FD-Bcm was therefore sought by MS analysis of the tryptic digest of FD-Bcm-modified Rho samples. The trypsin digestion experiments were conducted in two stages. In the first set of reactions, Rho was partially hydrolyzed using trypsin with subsequent conventional MS determination of the modified fragments. In a second series of experiments, the modified Rho sample was fully digested with trypsin to provide fragments amenable to tandem MS analysis, allowing the specific site of modification to be deduced.

von Hippel and co-workers (41) showed that partial tryptic digestion of Rho in the presence of poly(C) results in cleavage at only two sites, Lys283 and Arg128 or Lys130. In the present work, electrospray MS analysis of the product of partial tryptic digestion of native Rho gave precise estimations of mass for the fragments, enabling the definition of the second preferred cleavage site as Arg128, rather than Lys130 (data not shown). The conditions for partial hydrolysis were applied to the analysis of Rho following incubation with FD-Bcm (1 mM, 333-fold excess) and subsequent reduction. Initially, incubation was performed with poly(C), but no ATP, because high levels of FD-Bcm adduction and protein inactivation were observed under these conditions (Table II and Fig. 2). Fig. 3 shows the electrospray MS analysis of the partial hydrolysis product. Some intact protein remained, including a proportion of the modified protein. Products of partial hydrolysis were observed corresponding to residues 1–128 and 284–419; no satellite signals were present corresponding to modified analogues of these fragments. The cleavage product incorporating residues 1–283, however, was clearly present as a doublet separated by 407 Da (Fig. 3).

The reduced FD-Bcm-modified Rho product was then subjected to digestion with trypsin under conditions to achieve complete hydrolysis, and the products were separated by HPLC with UV absorbance detection. Comparison of the chromatogram with that obtained for the digest of unmodified Rho

**TABLE II**

| Entry | Rho | Poly(C) | ATP | FD-Bcm | ESI-MS | ATPase activity |
|-------|-----|---------|-----|--------|--------|-----------------|
|       | μM  | mM     | mM  | μM    |        | %              |
| 1     | 3   | 1      | 71  | 29     | 28     |
| 2     | 3   | 40     | 1   | 1      | 55     | 12              |
| 3     | 3   | 40     | 1   | 1      | 78     | 22              |
| 4     | 3   | 40     | 1   | 1      | 83     | 17              |
| 5     | 3   | 40     | 1   | 5      | 35     | 42              |
| 6     | 3   | 40     | 5   | 35     | 56     | 10              |
| 7     | 3   | 40     | 1   | 1      | 57     | 32              |
| 8     | 3   | 40     | 1   | 5      | 63     | 28              |

*a* The ratio was determined from the peak areas corresponding to the individual peaks in the maximum entropy-processed mass spectra.

*b* The percent ATPase activity of irreversibly modified FD-Bcm-Rho samples after dialysis. The corresponding value for a similarly treated Rho sample that did not contain FD-Bcm, poly(C), or ATP was 82%.

![Fig. 3. Mass spectrometric analysis of the product of partial tryptic hydrolysis of FD-Bcm-treated Rho.](http://www.jbc.org/)
indicated a single additional peak derived from the modified protein (data not shown). The tryptic peptides associated with this peak were collected and analyzed by electrospray MS (Fig. 4); two components were apparent with masses indicating the addition of 407 and 118 Da to the combined tryptic fragment, Gly-Leu-Ile-Val-Ala-Pro-Pro-Lys-Ala-Gly-Lys (residues 174–184). The peak of \( m/z \) 729.7 (corresponding to the doubly protonated molecule of the +407 adduct) was selected for collisional activation in a tandem MS experiment; Fig. 5 shows the product ion spectrum and the interpretation of the fragmentation pattern. The principal product ions corresponded to cleavage of peptide bonds with single charge retention on the N-terminal (b series) or C-terminal (y series) fragments. (The nomenclature adopted is the Biemann variant (35) of the original suggestion by Roepstorff and Fohlman (42).) The data were consistent with the modification of Lys181. The prominent *y6, *y7, and *y8 fragment ions, shifted by 407 Da in comparison with the masses expected for the native tryptic peptide, were accompanied by analogous cleavage products shifted by 118 Da. A prominent fragment was also observed corresponding to the doubly protonated native peptide plus 118 Da. These product ions are attributable to the apparently facile fragmentation of the FD-Bcm-derived moiety by cleavage of the exocyclic carbon-carbon bond. The observation of these ions in the tandem MS analysis of the adduct corresponding to the addition of 407 Da suggested that the presence of the [M + 118 + 2H]^{2+} (\( m/z \) 584.6; M corresponds to the native peptide) ion in the conventional electrospray spectrum (Fig. 4) was an artifact of MS analysis at the peptide level, no +118 adduct being observed during the analysis of the intact modified protein (Fig. 2). The product ion spectrum obtained by tandem MS analysis with selection of \( m/z \) 584.6 ([M + 118 + 2H]^{2+}) as precursor was consistent with Lys^{181} as the site of modification (data not shown). We also recorded product ion spectra for the corresponding triply charged ions in the conventional mass spectrum (Fig. 4); the data (not shown) were in accord with the conclusions based on analysis of the doubly charged analogues.

MS analysis of all the remaining HPLC fractions derived from tryptic hydrolysis of the modified protein yielded data for tryptic fragments accounting for more than 90% of the Rho protein sequence. Tryptic fragments not detected were exclusively those of 1–4 amino acids in length. No evidence was obtained for any other modified peptide, suggesting Lys^{181} as the sole site of selective modification.

In view of the protective effects provided by ATP during reductive amination of Rho with FD-Bcm in the presence of poly(C), as judged by ATPase activity and stoichiometry of covalent modification (Table II), we additionally investigated the site of FD-Bcm-Rho modification upon inclusion of ATP. Accordingly, Rho was incubated with FD-Bcm (1 mM) in the presence of poly(C) (40 nM) and ATP (1 mM). Subsequent reduction and sample work-up were performed as previously. Electrospray MS analysis of the intact protein indicated the presence of the native protein and of the singly modified protein, with relative abundance of approximately 7:1 (data not shown). Partial tryptic hydrolysis provided evidence for modification only of the fragment corresponding to residues 1–283, to an extent which paralleled that of the intact protein. Complete tryptic hydrolysis and HPLC analysis with UV absorbance detection showed the peak previously attributed to a modified tryptic fragment to be barely detectable (data not shown). Electrospray/tandem MS analysis of the corresponding HPLC fraction, however, enabled unequivocal characterization of this tryptic fragment and confirmed modification of Lys^{181} (data not shown). MS analyses of the other HPLC fractions afforded no evidence for other sites of modification.
When Rho was incubated with higher concentrations of FD-Bcm, evidence was obtained for the incorporation of a second FD-Bcm-derived moiety (Table II). Partial tryptic digestion and electrospray MS analysis (not shown) revealed modification of each hydrolysis product. Complete tryptic digestion with subsequent HPLC separation and MS analysis confirmed the modification of Lys181 but revealed no other site of adduct formation (data not shown). We interpret these data to indicate the existence of a single site of selective modification, with further adduct formation in the presence of high concentrations of FD-Bcm occurring in a nonspecific manner.

DISCUSSION

The reductive amination probe, 5α-(3-formylanilino)dihydrobicyclomycin (FD-Bcm), retained excellent inhibitory activity in Rho-dependent assays, providing preliminary evidence that this agent binds to the bicyclomycin (Bcm) binding pocket in Rho. This finding was supported by kinetic studies showing that FD-Bcm, like Bcm, was a noncompetitive, reversible inhibitor of Rho with respect to ATP and that Bcm and FD-Bcm competed for the same binding site in Rho. Treatment of Rho solutions containing FD-Bcm with NaBH₄ led to appreciable losses of Rho-dependent ATPase activity after dialysis, under various conditions. Co-incubation with poly(C) promoted adduction of FD-Bcm to Rho, whereas the presence of ATP diminished adduction. We speculate that the protective effects observed for ATP in the presence of poly(C) stem from an ATP-induced Rho conformational change (41, 43) that alters the spatial relationship of the reactive lysine residue in Rho with the C(5α) aryl aldehyde unit in FD-Bcm and prevents covalent adduction at some of the binding sites.

Mass spectrometric analysis of the product of incubation of Rho with FD-Bcm, with subsequent NaBH₄ reduction, provided evidence for the formation of an adduct with a mass consistent with reaction with a lysine residue. The extent of modification was inversely proportional to the residual Rho-ATPase activity. The stoichiometry of FD-Bcm-Rho covalent adduct formation observed in these experiments may be significant. In the presence of poly(C) (but without ATP), we observed high levels of permanent ATPase inactivation (88%) and the formation of a 1.2:1 ratio of free monomer to FD-Bcm-modified Rho. These findings indicate that the stoichiometry of the Bcm affinity probe to Rho is approximately three per hexameric Rho and suggest that full occupation of these three sites will lead to near complete loss of Rho-ATPase activity. Trypsin digestion of FD-Bcm-modified Rho samples followed by MS analyses of the peptide fragments indicated Lys¹⁸¹ to be a site of adduction, whether or not ATP was included in the incubation. Whereas the data are clear that this represents the only specific binding site when incubations are performed in the presence of poly(C) alone, the lesser extent of modification of Rho when incubation was performed in the additional presence of ATP leaves open the possibility of secondary binding sites not detected because of the low abundance of the corresponding modified tryptic peptides. The finding that covalent adduction of FD-Bcm to Rho proceeded at Lys¹⁸¹ was surprising. Previously, Platt and co-workers (44) reported that this residue was modified by an ATP-reductive amination probe (incorporating an aldehyde-containing moiety in the γ-position), thereby showing this amino acid to be near the terminal phosphate of Rho-bound ATP. Additionally, it was demonstrated that three
molecules of the probe bound per hexameric Rho. Similar results were reported by O and Stitt (45) following irradiation of the photocleavable probe, 8-azidoadenosine 5′-triphosphate, with Rho. These results, on first inspection, are surprising in view of our kinetic studies (Fig. 1) which demonstrated that FD-Bcm was a reversible, noncompetitive inhibitor of Rho-dependent ATPase activity with respect to ATP. Further consideration, however, enables us to explain these findings. Studies by Platt and co-workers (44) and Shigesada and co-workers (46, 47) have drawn attention to the similarity of the ATP binding sites in Rho,4 leading us to suggest that Lys181 is at the vertex of both the ATP- and the Bcm-binding sites. If this is the case, Lys181 within any given monomer can be potentially modified by FD-Bcm and the ATP reductive amination probe. Another explanation exists; the ATP and Bcm binding domains may not exist within the same monomer. Accordingly, FD-Bcm and the ATP reductive amination probes may target Lys181 in different monomers.

In summary, our investigation has led to the identification of a key amino acid residue at or near the Bcm binding domain within Rho. We suggest that this finding paves the way for future elaboration of this site. These studies have also clearly defined important unresolved issues concerning the stoichiometry of the Bcm-Rho complex and the relative distribution of the Bcm- and ATP-binding sites within Rho.

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