Identification of an RNA-binding Site in the ATP Binding Domain of Escherichia coli Rho by H$_2$O$_2$/Fe-EDTA Cleavage Protection Studies*

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Transcription factor Rho is a ring-shaped, homohexameric protein that causes transcript termination through actions on nascent RNAs that are coupled to ATP hydrolysis. The Rho polypeptide has a distinct RNA binding domain of known structure as well as an ATP binding domain for which a structure has been proposed based on homology modeling. Treatment of Rho with H$_2$O$_2$ in the presence of Fe-EDTA caused single-cut cleavage at a number of points that coincide with solvent-exposed loops in both the known and predicted structures, thereby providing support for the validity of the tertiary and quaternary structural models of Rho. The binding of ATP caused one distinct change in the cleavage pattern, a strong protection at a cleavage point in the P-loop of the ATP binding domain. Binding of RNA and single-stranded DNA (poly(dC)) caused strong protection at several accessible parts of the oligosaccharide/oligonucleotide binding (OB) fold in the RNA binding domain. RNA molecules but not DNA molecules also caused a strong, ATP-dependent protection at a cleavage site in the predicted Q-loop of the ATP binding domain. These results suggest that Rho has two distinct binding sites for RNA. Besides the site composed of multiples of the RNA binding domain, to which single-stranded DNA as well as RNA can bind, it has a separate, RNA-specific site on the Q-loop in the ATP binding domain. In the proposed quaternary structure of Rho, the Q-loops from the six subunits form the upper entrance to the hole in the ring-shaped hexamer through which the nascent transcript is translocated by actions coupled to ATP hydrolyses.

Transcript termination factor Rho is essential for orderly gene expression in many bacteria (1, 2). In Escherichia coli, Rho functions as a homohexamer of a 419-amino acid polypeptide (3–6). Both sequence and functional analyses have shown that Rho polypeptide has two distinct functional domains; one domain is the N-terminal RNA binding domain of residues 1–130 (RNA-BD),¹ and the other is C-terminal adenosine domain is the N-terminal RNA binding domain of residues 131–419 (ATP-BD). Sequence comparison of Rho with other proteins indicates that it has ribonucleoprotein-like motifs in the RNA-BD and several motifs that are characteristic of ATPases in the ATP-BD (reviewed in Ref. 7). High resolution structures of the RNA-BD by itself (8, 9) and in a complex with oligo(C) (10) reveal that it contains a classic OB-fold motif that can form a stable complex with RNA (11, 12). A model for the tertiary structure of the Rho ATP-BD has also been proposed based on the close sequence similarity of that part of Rho with the corresponding part of the α and β subunits of the F$_1$-ATPase (13).

A model for the quaternary structure of hexameric Rho has also been proposed (7, 13, 14) based on its sequence similarity and morphological resemblance to the F$_1$-ATPase (5, 6, 15). More recently, a low resolution, three-dimensional model of Rho has been constructed from the analysis of electron microscopic images of Rho stained with uranyl acetate (16). This model places the six RNA-BDs at one end of a ring-shaped structure with a C$_6$ symmetry. Although the precise orientation of the RNA binding clefts of the individual domain is uncertain, they are known to come together in the hexamer to form a single continuous cleft that is large enough to protect 60 nucleotides of poly(C) from cleavage by RNase A (4, 17).

In its function as a termination factor, Rho binds initially to an attachment site, called rut (for Rho utilization), on the nascent RNA (1). This interaction presumably involves a direct interaction between the rut sequence on RNA and the RNA-binding site that extends across several RNA-BDs in the hexamer. Termination then occurs as a result of subsequent interactions of Rho with the RNA. These interactions are coupled to ATP hydrolysis and permit Rho to translocate toward the 3' end of the transcript. Several mechanistic models have been proposed to account for the ATP-driven interaction of Rho with the RNA. In two of the models (18, 19), the interactions between Rho and RNA involve only contacts of the RNA with RNA-BDs in various subunits. The third model (7) involves contacts in the ATP-BD as well.

It has been proposed that Rho has two types of RNA-binding sites (20); one type is a primary site that is able to bind single-stranded polynucleotides (RNA or DNA), and the other type is a secondary site that is RNA-specific and that is coupled to interactions with ATP, the hydrolysis substrate. This proposal was based on enzymatic studies in which various polynucleotides were used to activate ATP hydrolysis (20). However, no direct demonstration has been made yet to identify a distinct RNA-specific site on Rho. The binding of poly(dC) by hexameric Rho is competitive with the binding of poly(C) (17), and oligo(dC) binds to the isolated RBA-BD almost as well as does oligo(C) (21). Thus the proposed primary site is very likely composed of the RNA-BD from multiple subunits. The putative secondary site, however, could also be located in RNA-BDs and coincide with conformational changes in some of the subunits, or alternatively, it could be located elsewhere within the Rho hexamer. The results in this paper address this question.

In this study, we used a protein footprinting method similar to...
to the one developed by Heyduk and Heyduk (22) to determine whether the binding of various ligands alters the portions of the Rho polypeptide that are exposed to solvent. In this method, mixtures of H$_2$O$_2$ with Fe-EDTA were used to cause partial chemical cleavage of the protein backbone. Because these reagents cleave proteins preferentially at solvent-accessible sites, this reaction was first used to test for predictions of the structural models. This method was then used to identify possible points of contact with various ligands. Both RNA and DNA caused protection at exposed parts of the OB fold in the RNA-BD, whereas only RNA caused the exposed Q-loop in the ATP-BD to be protected. These observations in conjunction with measurements of ATP hydrolysis suggest that the secondary binding site is at or near the Q-loop.

**EXPERIMENTAL PROCEDURES**

**Materials**—All nucleotides were purchased from Roche Molecular Biochemicals. Poly(C), poly(U), and poly(A) were purchased from Miles Laboratories, Inc. Poly(dC) was purchased from Amersham Pharmacia Biotech. Eight-residue-long oligocytidine [(Cp)$_8$] was purchased from Oligonucleotide, Inc. Oligonucleotide (A) and (A) (i-(Np)N) were prepared previously (20), and their lengths were confirmed on 20% polyacrylamide denaturing gel. Endoprotease Lys-C and 3,3-diaminobenzidine were products of Sigma. E. coli Rho protein was overexpressed from plasmid pCB111 and purified according to the previously published procedure (23). The peptide corresponding to Rho N-terminal sequence, MNLTELKNTPVSELIT, was synthesized in the laboratory of Dr. Roger Roeske, Department of Biochemistry, Indiana University, Indianapolis, IN, as previously described (24). The peptide corresponding to the C-terminal sequence, KTNDIFFEMMKRS, was synthesized by Research Genetics, Inc. Each peptide had an additional cysteine at the C-terminal end. They were reacted to Imject® maleimide-activated keyhole limpet hemocyanin (Pierce) following the manufacturer’s instructions. Rabbit antisera were raised against the two conjugated keyhole limpet hemocyanin (Pierce) following the manufacturer’s instructions. The collected sera were lyophilized for storage and redissolved in 50% glycerol before use. The secondary antibody, the horseradish peroxidase-conjugated goat anti-rabbit IgG, was purchased from Jackson Immunologic Research Laboratories, Inc.

The different λ cro RNA derivatives were synthesized in vitro by transcription of plasmid pFl2 using T7 RNA polymerase (25). Three DNA templates were prepared by linearizing plasmid pFl2 with either Tsp6, BstI, or BII restriction enzymes and purified as described (26). Burgess and Richardson (26). RNA transcripts from these DNA templates were then synthesized and purified as described by Gaq and Richardson (27).

H$_2$O$_2$/Fe-EDTA Protein Footprinting—Each 20-μl reaction mixture contained 0.56 μl Rho hexamer (3.2 μg), 40 μM Hepes-NaOH (pH 7.9), 150 μM sodium acetate. The concentrations of magnesium diions, adenosine nucleotides, and polynucleotides were specified in each experiment. The cleavage was initiated by adding freshly prepared 10× (NH$_4$)$_2$Fe(II)(SO$_4$)$_2$, EDTA (pH 8.0), and H$_2$O$_2$ to reach a final concentration of 1, 2, and 15 mM, respectively. Reactions were carried out at 37 °C for 5 min and terminated by adding 6 μl of 4× SDS loading buffer (200 mM Tris-Cl (pH 8.0), 8% sodium dodecyl sulfate, 0.4% bromphenol blue, 40% glycerol, 0.4 μM mercaptoethanol, and 0.2 μl thrombin). The products were then separated by electrophoresis on a polyacrylamide gel composed of a 5% stacking gel, a 10% spacer gel, and a 16.5% separation gel in a Tricine/SDS system (28).

**Internal Molecular Weight Marker**—Rho peptide fragments cleaved at Met residues were prepared by digestion of 2 μg of Rho in 20 μl of 10 mg/ml CNBr in 70% formic acid for 40 min at 22 °C. The reaction was terminated by adding 1 ml of deionized distilled water. The products were lyophilized and redissolved in 1× SDS loading buffer before use. Fragments cleaved at Lys residues were prepared by incubation of 2 μg of Rho with 25 ng of Lys-C in 20 μl of 50 mM Tris-Cl (pH 8.0) and 8 mM urea at 37 °C for 15 min.

**Western Blot**—After the electrophoresis, the separated polypeptides were transferred to nitrocellulose membranes (Schleicher & Schuell, BA 83, 0.2 μm). The electrophoretic transfer was carried out using a Genie electroblotter blotter (Idea Scientific) at 12 V for 2 h in the electroblotting buffer at 22 °C (25 mM Tris, 192 mM glycine, and 20% methanol, pre-chilled to 4 °C). All the subsequent procedures were done at room temperature. The membranes were air-dried and then treated for 30 min with blocking buffer (5% nonfat dry milk in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, and 1.4 mM KH$_2$PO$_4$). The membranes were incubated for 1.5 h with agitation in 30 ml of a solution containing antiserum raised against either the Rho N-terminal or C-terminal peptide conjugate diluted 1:250 in blocking buffer. The blots were then washed three times for 10 min each with phosphate-buffered saline and then incubated for another 1.5 h in a 1:500 dilution of the horseradish peroxidase-conjugated secondary antibody in the blocking buffer. After washing three times as before, the blots were developed in 100 ml of phosphate-buffered saline containing 50 mg 3,3-diaminobenzidine, 0.025% CoCl$_2$, 0.02% NiCl$_2$, and 0.03% H$_2$O$_2$.

**Data Analysis**—The blots were scanned and analyzed with IMAGEQUANT (Molecular Dynamics) to obtain the integrated intensity of each cleavage result. These data were then transferred to Microsoft EXCEL and quantified further. The differences in sample loading and cleavage efficiency were corrected based on methods described previously (29, 30). The percentage differences in cleavage intensity (protection) were calculated using the equation $I_{complex} - I_{rho} \times 100$, where $I_{complex}$ is the corrected intensity for Rho in complex with other ligands, and $I_{rho}$ is the corrected intensity for Rho alone or as indicated in each experiment. The percentage protection values presented were generated by averaging the results from at least three independent experiments.

**ATPase Assay**—The ATPase activities of Rho induced by the binding of various nucleic acid ligands were determined as described by Nawatzke and Richardson (31). 80 ng of Rho was incubated with 1 mM ATP at 37 °C for 5 min in 100 μl of the footprinting buffer (40 mM Hepes-NaOH (pH 7.9), 150 mM potassium acetate, 2 mM magnesium acetate) and RNA as indicated for each reaction.

**RESULTS**

**Cleavage of Rho by Hydrogen Peroxide and Fe-EDTA**—Treatment of proteins with a solution containing H$_2$O$_2$ and Fe(II)-EDTA causes partial cleavage of the polypeptide chain at solvent-exposed segments (22, 29, 30, 32, 33). The procedure is thus useful for probing the structure of proteins and for locating exposed regions that become blocked upon binding of ligands. To apply this approach to the structure and function of Rho factor, we first tested several conditions to find a reaction procedure that allowed partial cleavage of Rho during a relatively short incubation. Under our standard conditions, ~25% of the polypeptides were cleaved after 5 min (Fig. 1). This extent was low enough to avoid more than one cut per molecule (22, 34) but high enough to generate an array of distinct products. We found in the preliminary experiments that ascorbic acid, which was used in previously published procedures (22, 29, 30), was not needed and that reactions depended strongly on the H$_2$O$_2$ concentration. Also, although Fe(II) was used in all the experiments presented here, we obtained identical results with Fe(III). Thus we conclude that the cleavages are caused by actions of the iron-activated H$_2$O$_2$ on the proteins and probably not from hydroxyl radicals.

To locate the positions of the cleavage sites, we used an indirect immunological detection method (Western blot) to visualize the fragments ending at the N terminus or those ending at the C terminus, depending on the antiserum used.
Molecular weight markers were generated by residue-specific partial cleavage of Rho by CNBr, which targets methionine residues, or by Lys-C, which cleaves on the carboxyl group side of lysine residues. These residues are shown in the lanes labeled CNBr and LysC, with their approximate positions listed on the left of panels A and B. Combined Fe-EDTA cleavage profile of Rho polypeptide. Arbitrary intensity was plotted against the residue number. The profile on the left of the dashed line was from panel A and that on the right was from panel B. D, a diagram showing the secondary structural motifs of Rho. The motifs for the RNA-BD were from Ref. 9. The motifs of the model of ATP-BD (13) were determined by Secondary Reader using Protein Data Bank classification in Insight II, BIOSYM/Molecular Simulations. Dotted, filled, and open boxes represent α helices, β strands, and loops, respectively. The arrow indicates the trypsin cleavage point that defines the border between the RNA-BD and ATP-BD. Solid bars on top of the diagram mark the positions of the peaks in C.

To determine whether the interaction of ATP and other adenosine nucleotides has any effect on the cleavage pattern of Rho by the H$_2$O$_2$/Fe-EDTA complex, we conducted separate footprinting reactions in the presence of 1 mM ATP, ATPγS,
ADP, or AMPPNP. Fig. 3 shows the cleavage products detected with the N-terminal-specific antiserum. The only significant change in cleavage intensity observed was near residue 179, which is on the highly conserved P-loop, a part of the Rho polypeptide that is expected to have a close contact with the β and γ phosphate of ATP (15, 38, 39). The decrease in the cleavage intensity at this site upon ATP binding was also detected using the C-terminal-specific antiserum (data not shown). No significant change in cleavage intensity in other parts of Rho was evident with either antiserum. ATP and ATPγS gave much stronger protection than did ADP and AMP-PNP. The differences in the cleavage intensity that resulted from ligand binding compared with that of Rho alone were calculated. The averaged value of the percentage protection (\(I_{\text{complex}} - I_{\text{Rho}}/I_{\text{Rho}} \times 100\)) at residue 179 by each ligand is summarized in Fig. 3. These results provide direct evidence that ATP and ADP interacts with the P-loop of Rho without RNA present and that ATP protects the P-loop from the cleavage by the \(\text{H}_2\text{O}_2/\text{Fe-EDTA}\) complex better than does ADP.

**Protection of Rho RNA-BD by Various Polynucleotide Ligands**—We next used partial cleavages with \(\text{H}_2\text{O}_2/\text{Fe-EDTA}\) to probe for changes in Rho upon the addition of various synthetic RNA and DNA molecules. We first used the C-terminal-specific antiserum to detect the fragments arising from cleavage in the RNA-BD. Rho is known to bind to single-stranded C-rich RNA and DNA molecules (17, 21). A major part of this interaction is believed to occur in an extensive cleft formed by an arrangement of the RNA-BDs in hexameric Rho. When Rho and ATPγS were mixed with saturating amounts of poly(C) or poly(dC), the cleavage pattern obtained with \(\text{H}_2\text{O}_2\) and Fe-EDTA, there was a significant decrease in the cleavage of several of the readily accessible parts of the RNA-BD (Fig. 4A; compare lanes 2 and 3 with lane 1). These cleavage points that became protected were at or near residues 62, 65, 105 on the L12, n34 (part of L34), and L45, respectively. The percentage of protection at each site is summarized in Fig. 4A. Poly(dC) was just as effective as poly(C) in the extent of protection at the three sites, suggesting that the two polymers bind similarly in this domain. The extent of protection at each of the three positions by poly(C) and poly(dC) was about the same, suggesting a nearly uniform interaction at each of these different points. Poly(U), which binds to Rho much less tightly than Poly(C) (17), gave partial protection at the three points, whereas Poly(A), which has a very weak interaction with Rho (17), did not give significant protection (Fig. 4A, lanes 5 and 6).

Strong protection at these same elements in the RNA-BD was also observed when Rho was mixed with a full-length \(\lambda\) cro gene transcript, a natural RNA that is terminated by the action of Rho (Fig. 4C). Identical protection was achieved with a shorter \(\lambda\) cro transcript that extended just through most of the cro gene rut site (Fig. 4, B and C), but no protection was found with a partial transcript lacking the rut sequence (Fig. 4, B and C). Thus, these results with natural transcripts as well as those with synthetic polymers showed that extensive protection of loop regions in the RNA-BD correlates with high affinity binding of these polymers to Rho (17, 27).

Although poly(dC) binds as tightly as poly(C) to Rho, it does not activate ATPase hydrolysis. However, Rho will hydrolyze ATP when it is mixed with poly(dC) and oligo(C\(_b\)) (20), (Table I). This result has been interpreted as an indication that in addition to the primary binding site, which is not specific for RNA, there is a secondary binding site that is specific for RNA (20). To determine whether this possible RNA-specific site is within the RNA-BD, the extent of protection was examined for a mixture of poly(dC) with oligo(C\(_b\)). The result showed that the presence of oligo(C\(_b\)) did not alter the pattern of protection in the RNA-RBD when compared with the pattern with poly(dC) alone (Fig. 4A, compare lane 4 with lane 3). Thus, this experiment failed to reveal the presence of a possible RNA-specific component in the RNA-BD of Rho. This result essentially confirms the lack of differences in the protection by poly(C) and by poly(dC) in this region of Rho.

Another characteristic of the protection seen in the RNA-BD was the absence of dependence on ATP or ATPγS. No significant difference in the protection patterns afforded by all polynucleotides tested in the RNA-BD was observed when ATPγS or ATP was omitted from the reaction mixture (compare lanes 7 and 8 with lanes 2 and 3 in Fig. 4A and lanes 5 and 6).
Fig. 4. Both RNA and DNA protect multiple cleavage sites in the RNA-BD. Blots of separated cleaved proteins were stained using the anti-C-terminal serum. A, protection with synthetic homopolymer as ligands. Each reaction contained 3.2 μg of Rho, 3.2 μg of homopolymer (as indicated), 11 mM magnesium acetate, and 10 mM ATP for samples in lanes 1–6, and where indicated, with C8, Δ 0.6 pmol of cro derivatives where indicated. The extent of protection with poly(C) was about the same with poly(dC) with oligo(C8) mixture. Such protection was seen with poly(U) or with a mixture of poly(dC) and oligo(U8) (Fig. 5A, lanes 2 and 6). However, cleavage at that point was not protected by poly(dC) alone (Fig. 5A, lane 5). Thus, unlike the protections seen in the RNA-BD, the protection at residue 285 is specific for RNA. In the tertiary and the quaternary structural models of Rho, residue 285 is predicted to be on a solvent-exposed loop called the Q-loop (14).

Some of the other synthetic polymers also gave significant cleavage protection at residue 285, albeit less extensively than with poly(C) or the poly(dC)-oligo(C8) mixture. Such protection was seen with poly(U) or with a mixture of poly(dC) and oligo(U8) (Fig. 5A, lanes 3 and 7). When the effectiveness of these various polymers in activating ATP hydrolysis was measured (Table I), only those RNAs or combinations of DNA with oligonucleotides that activated ATP hydrolysis gave protection.

When a series of cro RNA derivatives was used as ligands in the footprinting assay under similar conditions, we could not observe significant protection at residue 285 when using ATPγS as the cofactor (Fig. 5B, lane 6–8) or when no adenine nucleotide was present (data not shown). However, we were able to detect a significant protection by cro-Taq RNA, the full-length cro transcript, when ATP was present (Fig. 5B, lane 2). Cro-BstX RNA, the cro derivative that ends near the 3′ end of the rut site, gave only borderline protection at residue 285, whereas cro-Bgl RNA gave no protection at this region under these conditions (Fig. 5B, lane 3 and 4). Again, the extent of protection with these cro RNA derivatives correlated with their abilities to activate ATP hydrolysis (Table I). Therefore, the segment of transcript that is 3′ to the rut site is needed to protect the Q-loop under these conditions, and the presence of ATP is critical to this observed protection pattern.

Although the protection of residue 285 by cro RNA is dependent upon the presence of ATP, this protection can be achieved by poly(C) alone (i.e. with no ATP or ATPγS; lane 9, Fig. 5A). The extent of protection with poly(C) was about the same with and without ATPγS. However, the protection of residue 285 by the mixture of oligo(C8) with poly(dC) was decreased from 37 to 11% in the absence of ATPγS (compare lane 6 to lane 11, Fig. 5A). Thus the protection in this instance correlates with the presence of RNA but not with ongoing ATP hydrolysis.

with lanes 2 and 3 in Fig. 4C). The only difference seen was the protection at P-loop region afforded by ATP or ATPγS as described earlier in Fig. 3.

RNA-specific Protection of Rho at the Q-loop Region in the ATP-BD—To determine whether RNA-specific protection could be detected in other regions of Rho, the gel-separated, partially cleaved complexes were visualized with the N-terminal-specific antiserum. When Rho was cleaved in the presence of various synthetic polymers, either poly(C) alone or a mixture of poly(dC) with oligo(C8) gave extensive protection at a prominent cleavage point near residue 285 (Fig. 5A, lanes 2 and 6). However, cleavage at that point was not protected by poly(dC) alone (Fig. 5A, lane 5). Thus, unlike the protections seen in the RNA-BD, the protection at residue 285 is specific for RNA. In the tertiary and the quaternary structural models of Rho, residue 285 is predicted to be on a solvent-exposed loop called the Q-loop (14).

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Table I

| Activator | ATPase activity (nmol min⁻¹ μg⁻¹) |
|----------|----------------------------------|
| Poly(C)  | 36.1 ± 0.7                      |
| Poly(U)  | 0.3 ± 0.1                       |
| Poly(A)  | <0.1                            |
| Poly(dC) | <0.1                            |
| Poly(dC) + C₈ | 34.3 ± 0.7                   |
| Poly(dC) + U₈ | 12 ± 0.1                    |
| Poly(dC) + A₈ | <0.1                        |
| cro-Taq (1–380) | 10.6 ± 0.2            |
| cro-BstX (1–276) | 1.7 ± 0.8                  |
| cro-Bgl (1–86)  | <0.1                        |

Each reaction mixture contained 80 ng of Rho, 80 ng of RNA or DNA homopolymers, 40 μM oligonucleotide and 0.6 pmol of cro derivatives where indicated.
Residues on the P-loop of Rho have been implicated in the binding of ATP through cross-linking studies using ATP analogs (40, 41) and by the functional properties of mutants with changes in conserved P-loop residues (40). In the structure of the complex of ATP with T7 gp4, a DNA helicase that is a structural homolog of Rho, Ser-314, which is its P-loop residue that is equivalent to Lys-181 in Rho, makes a hydrogen bond contact with an oxygen on the γ-phosphate of ATP (42). The formation of a similar hydrogen bond between Lys-181 in Rho and ATP or ATPγS could account for the change in conformation of the part of the P-loop that protects residues near Lys-181 from the cleavage with H₂O₂/Fe-EDTA. The absence of the γ-phosphate in ADP is likely to be the reason it gave much less protection than did ATP. Since the concentration of ADP used (1 mM) was well above the Kₚ for its complex with Rho (35), the lower protection with it cannot be a consequence of incomplete site occupancy. AMPPNP does have a γ-phosphate group, but it only gave partial protection. Again, because the concentration used was well above the Kₚ value for the Rho-AMPPNP complex (35), its binding site must have been fully occupied. Thus, the lack of protection must be a result of steric differences, possibly because of small differences in the bond lengths and geometries associated with the phosphoramidate link (43).

We observed that three segments of the Rho RNA-BD were protected by homopolymer ligands and by cro RNAs containing the rut site (Fig. 4). All three segments are located in the OB-fold region. The first protected region is on the loop that connects β strands 1 and 2 (residues 58–62). This loop contains several residues that are important for the strength and specificity of RNA binding. Martínez et al. (44) finds that F62S Rho was very defective in binding to RNA, whereas D60G Rho bound RNA more avidly than did wild type Rho but also with less discrimination. In the crystal structure of Rho RNA-BD with oligo(C₉), residue Leu-58 and Phe-62 create a hydrophobic pocket for the ribose of the oligonucleotide (10), and their interactions are consistent with our chemical cleavage protection results.

The second protected part is on the loop that connects β strands 3 and 4. In some other OB-fold proteins, residues on this loop are also involved in polynucleotide binding (9, 45). However, this region was not found to be involved in the interaction with the RNA ligand in the structure of the crystallized complex of RNA-BD with oligo(C₉) (10). This inconsistency may be a consequence of the special characteristics of the complex in the crystal. In that complex, two RNA-BDs share a single oligo(C₉) and, thus, has an average site occupancy of 4.5 C residues per RNA-BD. In hexameric Rho, the site occupancy is 10–13 C residues per RNA-BD (17, 46). Thus, the oligo(C₉)-RNA-BD complex may have formed without full binding site coverage in each of the subunits. On the other hand, the cleavage protection results are consistent with recent evidence that Arg-88 and Phe-89 display chemical shifts in the NMR spectrum of the isolated RNA-BD upon binding of C-rich oligonucleotides.²

The third protected region is on the loop connecting β strands 4 and 5 (residues 103–110). It has been suggested (10) that the side chains of Glu-108, Arg-109, and Tyr-110 on this loop are engaged in Van der Waals interactions with the base of a cytidine, whereas the main chain of this loop forms hydrogen bonds with the bases. The size of the cavity enclosed by L45 and β strand 3 is relatively restricted, which can easily shelter a pyrimidine but would be too small for a purine (9, 10). This could explain the preference Rho shows for binding pyrimidine nucleotides and was confirmed by the lack of protection by

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² T. K. Hitchins and G. S. Rule, personal communication.
polynucleotides protected in RNA-BDs are at disparate locations (Fig. 6), indicating that the binding site extends across all subunits. This result complements the finding that Rho protects a continuous 60-nucleotide segment of poly(C) from digestion with RNase A (4, 17). This RNaseA protection could come about from the RNA being bound in an extensive cleft made from the RNA-BDs of the six subunits arranged in a ring structure. Because the protection of cleavage by H2O2/Fe-EDTA in Rho RNA-BD was essentially the same with poly(dC) as with poly(C) and was the same in the presence of ATP or ATPγS as in their absence, this cleft must be the primary polynucleotide binding site.

Contrary to the relatively well defined primary RNA binding site, the location of the proposed secondary binding site has remained elusive. However, the protection by RNA at residue 285 on the Q-loop has several of the characteristics that are expected for the secondary binding site (20). First, this protection was RNA-specific. Both poly(C) and the combination of poly(dC) plus oligo(C8) gave strong protection at the Q-loop, whereas poly(dC) alone did not. Second, this protection showed the following base preference: C >> U > A. This preference correlates with the ability of oligonucleotides with these residues to activate ATP hydrolysis in conjunction with poly(dC) (Table I). Third, the extent of protection at the Q-loop by some RNAs was affected by the presence of ATP. The observation that the presence of ATPγS increases the protection by oligo(C8) in combination with poly(dC) is consistent with the earlier finding that the Km for oligo(C8) in activating ATP hydrolysis decreased when the concentration of ATP was increased (20). The protection of the Q-loop with cro RNAs was even more strictly dependent on ATP (Fig. 5B). On the other hand, poly(C) protected the Q-loop in the absence of ATP (Fig. 5A). This could be a consequence of its extremely high affinity for both the primary and secondary sites of Rho. Possibly, the binding of ATP may lower the stringency for an inherent cytidine requirement at the secondary site. This would be important if the secondary site is the one responsible for translocation, in which case it should be compatible with most RNA sequences.

Protection of the cleavage sites in the Q-loop could be caused by a direct physical contact with RNA or by a conformational change in the loop as a result of the interaction of RNA at another position. We favor the first interpretation for the following reason. In the Rho structural model, the Q-loops of the six subunits form the narrowest part of the central channel in the ring shaped hexamer (Fig. 6), and each Q-loop is right above another loop, called the R-loop, which can be cross-linked to RNA by a 20-Å photo-activable cross-linker (26). This cross-linking result indicates that RNA can pass through the channel. Hence, RNA is likely to make close contact with Q-loop residues because they are in the narrowest part of the channel.

The sequence alignment of Rho polypeptides encoded by the rho genes from several bacteria shows that the Q-loop region has several highly conserved residues (47). None of the known rho mutants changes residues in the Q-loop. We predict that mutational changes of some of these residues should affect the interactions of Rho with RNA that are coupled to ATP hydrolysis.

There have been two hypotheses on the nature of the two types of RNA binding sites on Rho. The first proposes that the two sites are different conformational states assumed by the RNA-BDs on different subunits (18, 19). The other proposes that the primary binding site is composed of multiple RNA-BDs and that there is an additional interaction site for RNA outside of the traditionally defined RNA-BD (7). Our findings in this study support the latter hypothesis and suggest that the secondary binding occurs in the ATP-BD and is closely coupled to ATP hydrolysis.

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