ATP and Other Nucleotides Stabilize the Rho−mRNA Complex†

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ABSTRACT: Transcription termination factor Rho from *Escherichia coli* is a protein that consists of a single 47 kDa protomeric unit that can form a hexameric structure. To determine whether active hexamers can form on an RNA by assembly of subunits, we measured the dependence of complex formation on the concentration of Rho protein in the presence and absence of various nucleotides and related the binding properties to association states determined from sedimentation properties. The results show that the presence of adenine nucleotides converts RNA binding from a multimeric process to a largely monomeric process and that the change correlates with the stabilization of multimers of Rho by the nucleotides. The experimental evidence also indicates that the hexameric form of Rho is stabilized slightly by binding to a transcript but that the protein on RNA is in equilibrium with nonhexameric forms. These results suggest that a Rho hexamer can form on a transcript by addition of subunits to a partial assembly, which means that the complex can consist of six subunits surrounding an RNA transcript as proposed in recent models for Rho action.

Rho factor, a protein found in *Escherichia coli* and other bacteria, acts to terminate transcription at certain sites on DNA templates (*1*). It consists of a 47 kDa protomer that forms a ring-shaped hexamer (*2*−*4*). Rho binds to a specific attachment site on a nascent transcript (called a *rut* site) and uses the energy from ATP hydrolysis to dissociate the transcript from its elongation complexes with RNA polymerase and DNA template (*5*). Because of its hexameric structure and its ability to couple ATP hydrolysis with the unwinding of RNA−DNA helices (*6*), Rho is considered a member of the hexameric helicase family of proteins (*7*, *8*).

A model that has been proposed for Rho action involves having the RNA on the 3′ side of the initial attachment site pass through the hole in the hexameric ring (*9*). The process of forming this structure is not known. One possible mechanism would be to have Rho thread onto the RNA from the 5′ end of the transcript. However, this mechanism is unlikely because Rho does not bind very tightly to an RNA lacking a *rut* site (*10*) and the *rut* site becomes available on the nascent transcript only after RNA polymerase has reached the region where transcripts are terminated (*11*). Also, in the cell, threading would be prevented by the presence of a translating ribosome on the transcript. Alternative ways for Rho to encircle an RNA would be to have the ring open temporarily between two subunits to let the RNA pass into the center or to assemble the Rho hexamer on the RNA from free subunits. To understand how Rho acts to cause termination, the mode with which it binds to RNA needs to be elucidated.

Previous studies on the association states of Rho have shown that the formation of the hexamer depends on the concentration of the protein and is influenced by the presence of ATP and the ionic conditions (*12*, *13*). The effect of ATP is quite dramatic. When Rho is at a concentration of ∼1.5 μg/mL, the apoprotein is monomeric while the complex with ATP is largely hexameric. This effect on multimerization should presumably influence how Rho binds to RNA. Yet in the one study in which binding of Rho to a transcript was measured in the presence and absence of ATP, no major differences were observed (*10*). To reevaluate that finding and to determine whether the interaction of Rho with a transcript can stabilize the hexameric form, we used filter-retention assays and zone sedimentation methods to measure complexes of Rho with two forms of the bacteriophage λ *cro* RNA. One form is the full-length transcript ending in the tR1 terminator. The other is a 61 nt[1] segment of that RNA consisting of a transcript of the *rut* region of tR1. We find that ATP does have a very strong effect on the binding of Rho to these RNAs that can be ascribed to the influence of ATP on the association state of Rho and that a Rho hexamer can form on a transcript by assembly of subunits.

EXPERIMENTAL PROCEDURES

Biochemicals and Enzymes. Unlabeled nucleotides were purchased from Roche Molecular Biochemicals. [γ-^{32}P]ATP was from ICN Pharmaceuticals. Restriction enzymes and T4 polynucleotide kinase were from New England Biolabs; T4 RNA ligase was from Pharmacia Biotech; ampicillin and dithiothreitol were from Sigma. Sterox was purchased from Bacharach, Inc. T7 RNA polymerase and Rho were purified by Lislott Richardson as described in Tabor and Richardson (*14*) and Nowatzke et al. (*15*), respectively.

Plasmid pIF2 is described in Faus and Richardson (*16*). Plasmid pB3, constructed by Dr. William Scott, contains

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[1] Abbreviations: AMPPNP, 5′-adenylyl imidodiphosphate; AcBSA, acetylated bovine serum albumin; bp, base pair(s); EDTA, (ethylenedinitrilo)tetraacetic acid; nt, nucleotide(s); Tris, tris(hydroxymethyl)-aminomethane.

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DNA bp[1] 213−310 of the bacteriophage λ *cro* gene (start point of transcription is bp +1) flanked by DNA sequences encoding hammerhead ribozymes between the *Xba*I and *Hin*dIII restriction sites in vector pUC18. The sequence of the pB3 insert (with the λ *cro* DNA sequence underlined) is 5′-CTAGAATGCTACTGATGAGGTTCGCCGAAACGTTCGCGTCTAGCATAAATAACCCCGCTCTTACACATTCCAGCCCTGAAAAAGGGCATCAAATTAAACCACACCTATGGTGTATGCATTTATTTGCATACATTCAATCTCTACCGAAAGGTACTGATGAGGTTCGCCGAAACGTTGCGTTA. pB3 was maintained in *E. coli* DH5αF′. Plasmids isolated by the alkaline lysis method (*17*) were treated for 1 h at 37 °C with 50 μg/mL RNase A, and then extracted once with water-saturated phenol and once with a 1:1 mixture of water-saturated phenol and chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 2 M ammonium acetate and 2 volumes of ethanol and resuspended in TE (10 mM Tris-HCl, pH 8, 1 mM EDTA).

DNA templates used for transcription were digested sequentially with a restriction enzyme and with proteinase K, and then extracted sequentially with equal volumes of H_2O-saturated phenol, a 1:1 mixture of H_2O-saturated phenol and chloroform/isoamyl alcohol (24:1), and 3 times with chloroform/isoamyl alcohol (24:1). After ethanol precipitation, the DNA was dissolved in TE. Full-length λ *cro* RNA was prepared as described by Faus and Richardson (*16*) by transcription of pIF2 cleaved with *Taq*I. B3′ RNA was prepared by transcription of pB3 that had been digested with *Bst*XI. The reaction mixture for pB3 transcription contained 40 mM Tris-HCl, pH 8, 30 mM $MgCl_2$, 5 μg of digested plasmid, 4 mM ATP, 4 mM CTP, 4 mM UTP, 4 mM GTP, 10 μg of T7 phage RNA polymerase, 2 mM spermidine, and 250 mM NaOH in a 100 μL reaction (*18*). The reaction mixture was incubated at 37° C for 4 h. After ethanol precipitation, the RNA was purified by electrophoresis on a 6% (w/v) polyacrylamide gel containing 8 M urea and TBE (90 mM Tris−borate, 2 mM EDTA). Bands containing the correct RNA were visualized by ultraviolet shadowing and excised. The gel slices were soaked in solutions containing 200 μL of water-saturated phenol, 200 μL of chloroform/isoamyl alcohol (24:1), and 200 μL of TES (50 mM Tris-HCl, pH 8, 2 mM EDTA, 0.3 M sodium acetate) for 12 h at 4 °C to elute the RNA. The aqueous layer was then removed, and after two rounds of ethanol precipitation, the pellets were dried and resuspended in 20 μL of TE. The concentrations of RNA were determined by measuring A_{260}. ^{32}P-labeled RNA was prepared by ligation of [5′-^{32}P]pCp to the 3′ end by the action of RNA ligase as described by Ceruzzi et al. (*10*).

Rho−RNA Binding Assays. Reaction mixtures (100 μL) contained either KGlu binding buffer [40 mM Tris−acetate, pH 8, 4 mM magnesium acetate, 150 mM potassium glutamate (adjusted to pH 8 with KOH), 0.1 mM dithiothreitol, 0.1 mM EDTA] or KCl binding buffer (40 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 50 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA), with 0.25 mg/mL acetylated bovine serum albumin (AcBSA), 0.1 nM [^{32}P]B3′ RNA, the indicated amount of Rho, and, where indicated, 1 mM ATP, 1 mM ADP, or 1 mM AMPPNP. The reaction mixtures were incubated at 37 °C for 10 min, and 80 μL from each was filtered through a 13 mm Schleicher & Schuell BA85 nitrocellulose filter. After being washed twice with 250 μL of binding buffer, the filters were dried and placed in 2 mL of Econoscint O (National Diagnostics), and the radioactivity was measured with a Packard Model 1600TR Scintillation Analyzer.

The binding assay measures the fraction of total RNA retained:

$$f_R = e\left(\frac{[C]}{[R] + [C]}\right)$$

where *e* is the retention efficiency, C is the Rho−RNA complex, and R is the free RNA. For the binding of a molecule of Rho (L, ligand) to one molecule of RNA,

$$K_d = \frac{[R][L]}{[C]}$$

and, by substitution:

$$f_R = \frac{e[L]}{K_d + [L]} \quad (1)$$

In some cases, the binding of Rho and RNA might involve a cooperative interaction of several subunits binding to a single RNA. If we assume an "all or none" mode of binding of *n* subunits, in the reaction

$$nL + R \leftrightarrow C$$

the equilibrium constant for this multiple subunit interaction would be

$$K_{nd} = \frac{[L]^n[R]}{[C]}$$

Expression in terms of the fraction of RNA bound as a function of *n* and L gives a form of the Hill equation:

$$f_R = \frac{e[L]^n}{K_{nd} + [L]^n} \quad (2)$$

where *n* is the Hill cooperativity constant.

Measurement of Dissociation Rates: Method 1: Competition Assay. Reaction mixtures (500 μL) in KCl binding buffer with nucleotides as indicated, and 0.25 mg/mL AcBSA, 0.1 nM [^{32}P]RNA (as indicated), and Rho at a concentration 1.5 times that needed for half-maximal binding in the solution used were incubated at 37 °C for 10 min before 1 μL of 2 μM unlabeled RNA of the same kind was added (final concentration: 4 nM). Samples of 50 μL taken every 3 min over a time course of 12 min were passed through a 13 mm Schleicher & Schuell nitrocellulose filter. After being washed twice with 250 μL of binding buffer, the filters were dried and assayed for radioactivity. The Rho concentrations used were 90, 12, 23, and 35 ng/mL, respectively, for assays carried out in the presence of no nucleotides, 1 mM ATP, 1 mM ADP, and 1 mM AMPPNP.

Method 2: Dilution Dissociation Assays. Reaction mixtures (200 μL) consisted of KGlu binding buffer or KCl binding buffer with nucleotides as indicated and 0.25 mg/mL AcBSA, 0.1 nM [^{32}P]RNA, and Rho at a concentration 1.5 times that needed for half-maximal binding in the solution used (0.012−1.2 μg/mL). After incubation at 37 °C for 10 min,

1.8 mL of the corresponding binding buffer was added to each mixture to dilute Rho. During further incubation at 25 °C, 350 μL samples were removed at 0, 3, 6, 9, and 12 min and filtered through a 13 mm Schleicher & Schuell nitrocellulose filter. After washing once with 350 μL of binding buffer, the filters were dried and assayed for radioactivity.

Determinations of Sedimentation Coefficients. Samples containing 1.4 μg/mL Rho, 1 mM adenine nucleotide, and RNA, as indicated, in 200 μL of sedimentation buffer (40 mM Tris−acetate, pH 8, 4 mM magnesium acetate, 150 mM potassium glutamate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.25 mg/mL AcBSA) with 5% glycerol were layered on top of 5 mL of 10−30% linear glycerol gradients in sedimentation buffer. For samples containing ATP or ADP, the corresponding nucleotide was also present at 1 mM concentration in the glycerol gradient. For markers, a 200 μL sample of 80 μg of catalase, 30 μg of alkaline phosphatase, and 225 μg of hemoglobin was layered on a glycerol gradient and centrifuged in a separate tube. After centrifugation at 45 000 rpm for 15 h at 4 °C in a Beckman SW50.1 rotor, 250 μL fractions were collected by pumping the gradient from the top. For ATPase assays, 25 μL of each fraction was added to 100 μL of ATPase solution containing 1 μg/mL poly(C). P_i released was measured by the malachite green method, as described in Nowatzke et al. (*15*). Hemoglobin was detected by measuring A_{410}. Alkaline phosphatase activity was detected by measuring the increase in A_{410} due to conversion of 3 mM *p*-nitrophenyl phosphate to *p*-nitrophenol and P_i in 600 mM Tris-HCl, pH 8. Catalase activity was detected by the presence of bubbling when 10 μL of gradient fraction was mixed with 50 μL of 60 mM H_2O_2, 50 mM Tris-HCl, pH 6.8.

RESULTS

RNA Binding Assays. A long-term goal is to determine the structure of hexameric Rho bound to a natural attachment site from a mRNA. One such site, the *rut* RNA segment of λ *cro* RNA, serves as a model for such studies. An RNA consisting of the *rut* sequence from nt 216 to nt 276 of λ *cro* RNA was prepared by transcription of pB3 DNA that had been cleaved with *Bst*XI. The primary transcript contains a self-cleaving hammerhead ribozyme at its 5′ end. The 3′ cleavage product, B3′ RNA, was isolated by gel electrophoresis and used for binding and sedimentation studies.

To measure binding, a filter retention assay was used to determine the fraction of RNA retained in complexes as a function of protein concentration. When Rho was mixed with a limiting amount of RNA in the absence of ATP in a solution containing 0.15 M potassium glutamate and 4 mM magnesium acetate [ionic conditions that are optimal for Rho termination activity (*19*)], the increase in the fraction of RNA in the complex was not proportional to the amount of Rho at the lowest concentrations as would be expected for a normal monomeric binding reaction (Figure 1). Instead, the increase in the fraction of RNA in the complex showed a multimeric dependence. Hence, the data were fit to an equation for a multimeric binding reaction (eq 2 under Experimental Procedures). The curve that is shown is the best-fit of the data to that equation. The parameters K_{nd} and n for that curve are presented in Table 1. Because the conditions affect the molecular form of Rho, the results are presented with weight concentration units, and the value of K_{nd} is given in weight units to the power of n.

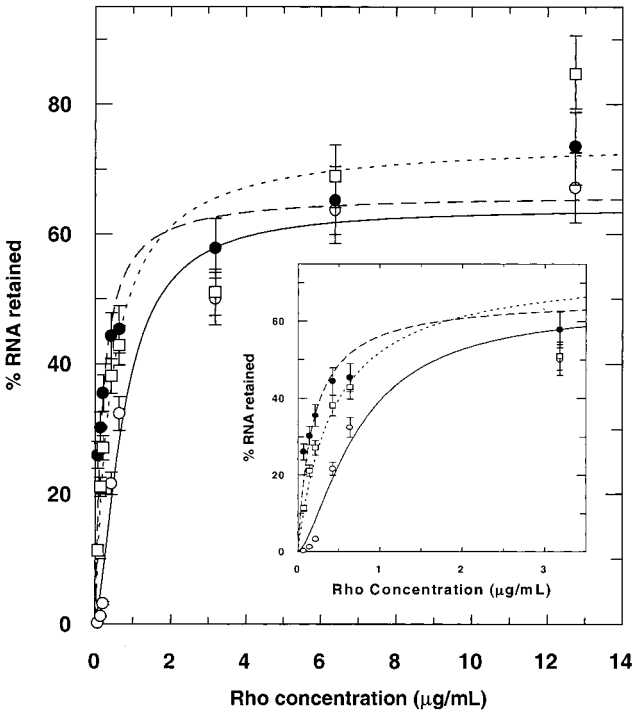

FIGURE 1: Plots of percent B3′ RNA retained on nitrocellulose filters as a function of Rho concentration. All samples contain 0.1 nM [^{32}P]B3′ RNA and the indicated concentrations of Rho protein in the KGlu binding buffer with no nucleotide (○), with 1 mM ATP (●), or with 1 mM ADP (□). The inset shows the lowest concentration range on an expanded scale.

When the binding reactions were performed with ATP present, the results were very different, with the increase in the fraction of RNA bound showing a monomeric dependence on Rho concentration (Figure 1). The data fit well to the equation for the monomeric binding reaction (eq 1), as shown by the associated curve. The K_d parameter for this curve is presented in Table 1. The values of e, the efficiency of retention, for all the binding reactions were in the range of 60−70%, which is typical for the filter retention assay (*16*, *20*), and are not presented in the table. We conclude from these results that the presence of ATP has a strong effect on the mode of binding of Rho to RNA.

To determine whether the effect of ATP on binding is dependent on the hydrolysis reaction catalyzed by Rho−RNA complexes, the binding reactions were also performed with two other adenine nucleotides that can bind to Rho but are not substrates for hydrolysis. When ADP or AMPPNP was present, the binding was monomeric. The data for ADP are shown in Figure 1 along with the best-fit curve. The data for AMPPNP were very similar and are not shown. The K_d parameters for both are presented in Table 1. These results indicate that the change in the binding mode of Rho to RNA is due to the interaction of Rho with a nucleotide and not to some reaction that is coupled to the hydrolysis of ATP by Rho.

This effect of ATP and other adenine nucleotides on the binding of Rho and B3′ RNA was surprising because a previous study on the binding of Rho to full-length λ *cro* RNA had not revealed any effect of ATP on the binding reaction (*10*). Because of this discrepancy, we also reinves-

Table 1: Values of Apparent Dissociation Constants from Best-Fits of Binding Data to Binding Equations

RNA and conditions	no nucleotides[a]			K_d (μg/mL)		
	K_{nd} (μg/mL)^n	n	$\overline{K_d}$ ($\mu g/mL$)	+ATP[b]	+ADP[b]	+AMPPNP[b]
B3′ RNA, 150 mM KGlu	0.63 ± 0.33	1.5	0.74 ± 0.49	0.18 ± 0.04	0.44 ± 0.13	0.33 ± 0.08
B3′ RNA, 50 mM KCl	0.12 ± 0.06	1.6	0.268 ± 0.174	0.035 ± 0.003	0.081 ± 0.022	0.079 ± 0.016
full-length *cro* RNA, 50 mM KCl	0.003 ± 0.001	2.0	0.057 ± 0.040	0.008 ± 0.001	0.015 ± 0.005	0.023 ± 0.006

[a] Values of K_{nd} and n were from best-fits of binding data to eq 2. $\overline{K_d}$ is the nth root of K_{nd}. [b] Values of K_d were from best-fits of binding data to eq 1. A weight concentration of 1 μg/mL is equivalent to 3.54 nM for hexameric Rho and 21.2 nM for monomeric Rho.

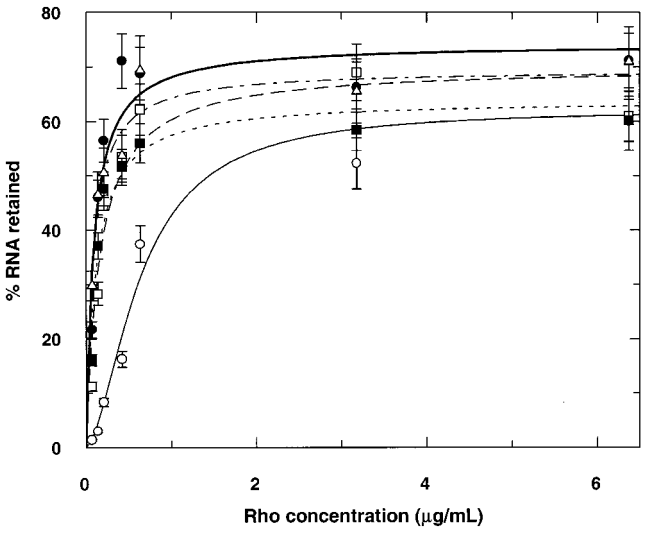

FIGURE 2: Binding of Rho to λ *cro* RNA in the presence of GTP, dATP, and 10 μM ATP is monomeric. All samples contained 0.1 nM ^{32}P-labeled λ *cro* RNA and the indicated concentrations of Rho protein in the KCl binding buffer with no nucleotide (○), with 1 mM ATP (●), with 1 mM GTP (■), with 1 mM dATP (△), and with 10 μM ATP (□).

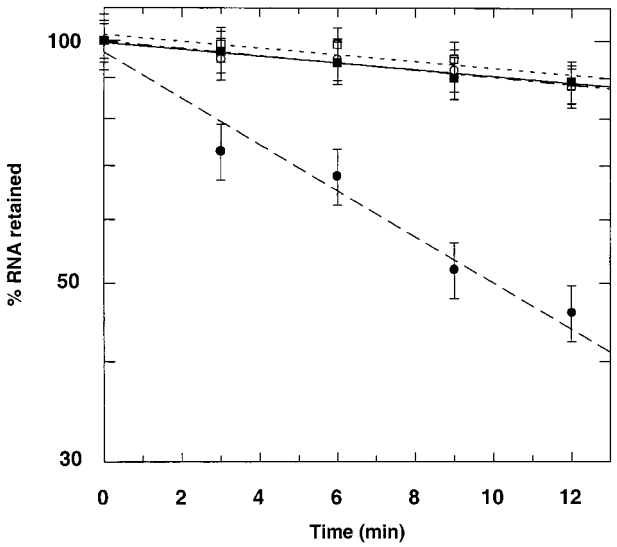

FIGURE 3: Stability of Rho−*cro* RNA complexes. The amounts of complexes of Rho with [^{32}P]*cro* RNA in the KCl binding buffer were measured at different times of incubation under four sets of conditions: with no ATP and no RNA (○); with no ATP and 4 nM unlabeled *cro* RNA (●); with 1 mM ATP and no RNA (□); with 1 mM ATP and 4 nM unlabeled *cro* RNA (■).

tigated the binding of Rho to a full-length *cro* RNA and to B3′ RNA in the ionic solutions used in the previous studies. With these conditions as well, the binding of Rho to these RNAs was multimeric in the absence of adenine nucleotides and monomeric in the presence of the nucleotides (data not shown). In each case, the binding data were fit to their respectively appropriate binding equation, and the parameters for the best-fit curves are presented in Table 1. Thus, the binding of Rho to the full-length *cro* RNA even in the binding buffer with 0.05 M KCl is affected by the presence of ATP and other adenine nucleotides. One likely reason this effect was not uncovered in the earlier studies is that the RNAs used then were not purified by gel electrophoresis and therefore might have been contaminated with nucleoside triphosphates.

Rho factor has a broad nucleoside triphosphate substrate specificity; it can catalyze the hydrolysis of all four NTPs and dATP (*21*, *22*). When we measured the binding of Rho to full-length *cro* RNA in the presence of two other representative substrates, GTP and dATP, the binding curves (Figure 2) were almost identical to the curve with ATP, thus suggesting that the effect is due to the binding of nucleotides to the sites that are used for NTP hydrolysis.

The concentration of nucleotides that were used for these studies, 1 mM, is similar to that used for most assays for Rho function in termination (*23*). However, because Rho has a very high affinity for ATP (*23*), much lower levels should be sufficient to saturate their effects related to binding. Indeed, when solutions containing as little as 10 μM ATP were used, the binding of Rho to λ *cro* RNA followed a monomeric dependence on Rho concentration (Figure 2). For this reason, a contamination of RNA with low levels of NTPs could greatly affect the outcome of the binding experiments.

Stability of Rho−RNA Complexes. To determine if the presence of adenine nucleotides is stabilizing the Rho−RNA complex, we measured dissociation of the complexes in a competition assay, in which excess unlabeled *cro* RNA was added to preformed complexes of Rho with labeled RNA and samples were filtered at intervals over a time course of 12 min. The results (Figure 3) show that the complexes dissociate much more readily in the absence of adenine nucleotides than in their presence. The first-order rate constants for dissociation in these experiments as well as others (not shown) using 20- and 10-fold excess unlabeled RNA as well as no RNA were measured by curve-fitting. Although we found no concentration dependence of the rates on the competing RNA, there was a measurable loss of complexes in the absence of a competitor, presumably from inactivation (*24*). The first-order rate constants for dissociation corrected for the rate of inactivation are presented in Table 2. Thus, in the absence of adenine nucleotides, the complexes of Rho on RNA dissociate 6.5 times faster than those in the presence of ATP. To investigate whether the hydrolysis of ATP is involved in the stabilization, rates of dissociation were also measured in the presence of 1 mM ADP and 1 mM AMPPNP. The results show that these

Table 2: First-Order Rate Constants for Dissociation of Rho−RNA Complexes

RNA and conditions	k (ks^{-1})			
	no nucleotides	+ATP	+ADP	+AMPPNP
full-length *cro* RNA, 50 mM KCl[a]	1.10 ± 0.11	0.17 ± 0.02	0.22 ± 0.05	0.43 ± 0.1
full-length *cro* RNA, 50 mM KCl[b]	1.12 ± 0.09	0.30 ± 0.04	0.17 ± 0.05	0.22 ± 0.06
B3′ RNA, 50 mM KCl[b]	3.93 ± 0.25	0.92 ± 0.13	0.87 ± 0.04	0.93 ± 0.16
B3′ RNA, 150 mM KGlu[b]	0.82 ± 0.07	0.15 ± 0.02	0.18 ± 0.05	0.17 ± 0.02

[a] Rate constants were determined by the competition procedure and are corrected for the rate of inactivation, which had a first-order rate constant (k_{inac}) of 0.04 ks^{-1}. [b] Rate constants were determined by the dilution procedure.

nonsubstrate nucleotides were similar to ATP in their effect on the stability of the Rho−RNA complex (Table 2).

An alternative method was also used to measure dissociation rates. Rho−RNA complexes were allowed to form in the presence and absence of nucleotides with an amount of Rho that was 1.5-fold greater than the amount of Rho that gave half-saturation of binding for the given condition. The reaction mixtures were then diluted 10-fold with binding buffer to reduce the overall concentration of Rho to a value that gave very little binding, and the amounts of RNA still bound to Rho were measured at different times. The rate constants determined using this procedure with two RNAs and under two conditions are presented in Table 2. The results with the full-length *cro* RNA in 0.05 M KCl were very similar to those determined by the other procedure. The complexes with B3′ RNA, though less stable than those with the wild-type *cro* RNA, were still significantly more stable in the presence of nucleotides. Also, the complexes with B3′ RNA were significantly more stable in the solution with 0.15 M potassium glutamate than in the one with 0.05 M KCl and again were stabilized further in the presence of nucleotides. We therefore conclude that adenine nucleotides stabilize the Rho−RNA complex.

Sedimentation Assays. To determine whether binding to a 'minimal *rut*' RNA induces the formation of hexameric Rho and to correlate the binding properties with the states of oligomerization, the sedimentation rates of Rho were measured by zonal centrifugation on glycerol gradients. The extent of migration of Rho was determined by an assay for poly(C)-dependent ATPase activity in the fractions, and sedimentation coefficients were determined from comparison of the migration of marker proteins. When 1.4 μg/mL Rho was layered on a gradient in 0.15 M potassium glutamate binding buffer and subjected to centrifugation, it migrated to a point in the gradient corresponding to a sedimentation coefficient of 4.3 S (Figure 4A). In the presence of B3′ RNA and no adenine nucleotides, Rho sedimented to the same point. The distribution of the labeled B3′ RNA in the same gradient (Figure 4B) indicated, by comparison with the distribution of the free RNA, that it formed a complex with Rho even though there was no apparent change in the sedimentation rate of Rho. Because about half of the RNA comigrated with the molar excess of monomeric Rho in this gradient, we estimate that the K_d for the complex of the monomer with RNA is 20 nM.

In the presence of 1 mM ATP or in the presence of 1 mM ADP, Rho sedimented to a point in the gradient corresponding to 8.3 S, indicating that it had formed a multimeric structure. With 1 mM ATP and a molar excess of B3′ RNA, Rho sedimented to a point in the gradient corresponding to 9.3 S, and about half the RNA comigrated with Rho. These

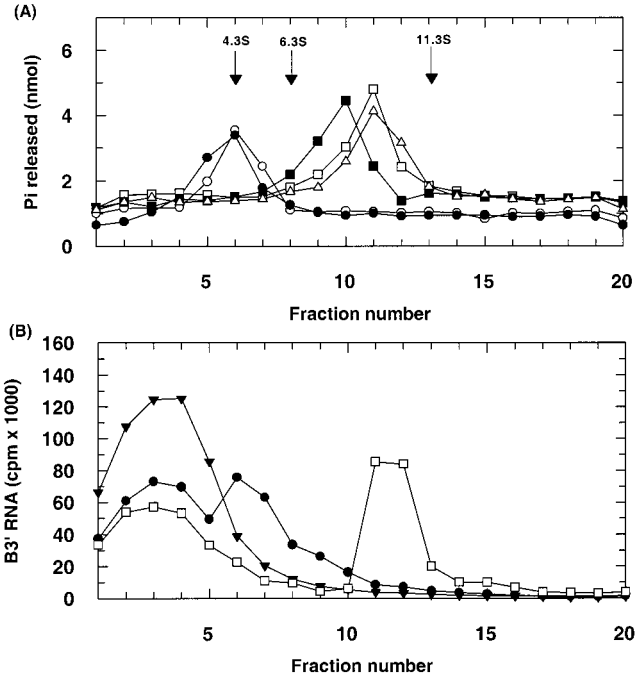

FIGURE 4: Sedimentation of Rho and complexes of Rho with the 62 nt B3′ RNA. Solutions (0.2 mL) contained 1.4 μg/mL Rho (1 pmol of hexamers) in the KGlu binding buffer with 1 mM ATP, 1 mM ADP, and 4 pmol of [^{32}P]B3′ RNA, where indicated. The samples containing ATP or ADP were layered on gradients containing the same amount of those respective nucleotides. Panel A: distribution of Rho, indicated by P_i released in ATPase assays. Panel B: distribution of [^{32}P]B3′ RNA. Symbol key: Rho alone (○); with B3′ RNA (●); with ATP (■); with ATP and B3′ RNA (□); with ADP and B3′ RNA (△) (distribution of RNA not shown); (▼) B3′ RNA alone.

results confirm that adenine nucleotides have a strong effect on the oligomeric structure of Rho at the low concentrations used for the binding studies. In addition, they show that the binding of Rho to its attachment site on a mRNA molecule has only small effects on the state of oligomerization.

DISCUSSION

Rho is known to form a hexameric structure and apparently exists as such when it is acting on a transcript to effect its termination. However, the hexamer readily dissociates. Indeed, our sedimentation studies confirmed that in the concentration range used for the binding studies (~1 μg/mL) Rho sediments at 4.3 S in the absence of ATP, indicating that it is primarily monomeric. Thus, the formation of a stable complex with *cro* RNA would depend either on the preassociation of subunits to form a hexamer or on the cooperative assembly of subunits on RNA. Both processes would appear to show a multimeric dependence on Rho concentration. The fact that B3′ RNA comigrated with Rho as a complex with

a sedimentation coefficient of 4.3 S indicates that a single monomer can bind to RNA. This finding suggests that binding can occur by an assembly of subunits on RNA.

Our sedimentation studies confirm that adenine nucleotides stabilize the multimeric forms of Rho. In the concentration range used for the binding studies, Rho sedimented at 8.3 S, which is lower than the 11.2S value for the hexamer (*13*). However, since hexamers are known to be present in solutions that show this reduced *s* value (*12*), the measured value of 8.3 S is evidently an average for a protein that is in equilibrium between the hexamer, smaller oligomers, and monomers. Thus, even though the data for the amounts of RNA bound to Rho in the presence of ATP fit well to the equation for the binding of a single unit to the RNA, the data were not good enough to rule out a binding mode that would involve addition of free subunits to a partial assembly of hexamers. Clearly, by stabilizing the multimeric forms of Rho, ATP affects greatly the concentration dependence of the formation of complexes with cro RNA.

The complex of Rho with B3′ RNA sedimented at 9.3 S with ATP present. Although this is slower than expected for a globular complex with a 20 kDa RNA added to the 283 kDa Rho hexamer, it is faster than the 8.8S value expected for the addition of 20 kDa to the average mass of a globular protein with a sedimentation coefficient of 8.3 S. This result suggests that even in the presence of ATP the complex of Rho with B3′ RNA has the sedimentation coefficient expected for a mixture that includes hexamers in equilibrium with smaller oligomers and monomers, but a mixture with a slight enrichment of the amount of hexamer present over that without the RNA. Thus, when bound to RNA, the hexamer is stabilized slightly but is in rapid equilibrium with free subunits.

Because of the complexity of the binding modes, we presented the binding data as a function of protein weight concentration rather than molar concentration. In previous studies from this laboratory, it was assumed that Rho was binding as a hexamer and the *K* values were presented in molar units for the Rho hexamer (*16*). For comparative purposes, the corresponding molar K_d value for our best-fit curve is 30 pM for the full-length *cro* RNA with ATP in 0.05 M KCl. This value is close to the 20 pM value reported by Steinmetz and Platt (*25*), who measured binding to full-length *cro* RNA in their helicase reaction buffer, which contained ATP. It is significantly lower than the 1.25 nM value reported by Faus and Richardson for binding in the absence of ATP. Faus and Richardson did not make measurements in the presence of ATP, and their curves were fit to an equation for a monomeric reaction using data from the higher end of the binding curve, where cooperative effects that are now evident are less apparent. Using that procedure, we replicated the binding affinity value reported by Faus and Richardson (data not shown).

The K_{nd} constants for the multimeric binding equation that gave the best fits of binding data measured in the absence of adenine nucleotides are not easily interpreted at the molecular level. Since free Rho under those conditions was monomeric, the binding represents a cooperative assembly in which the first subunit binds to RNA with a very low affinity while the subsequent subunits bind with higher affinity. The constant for the multimeric binding equation has its units to the *n*th power and is thus the product of *n* monomeric constants. The *n*th root of this constant would represent a geometric mean of those monomeric constants. These mean values ($\overline{K_d}$) are presented in Table 1. The corresponding molar value of this average dissociation constant for the full-length *cro* RNA in 0.05 M KCl based on the concentration of Rho monomers is 1.2 nM.

For B3′ RNA in 150 mM KGlu, the molar value based on Rho monomers would be 16 nM, which is close to the K_d for the interaction of a single monomer with that RNA that was estimated from the cosedimentation.

The value of *n* depends on the number of independent RNA binding sites in the Rho hexamer and on the degree of cooperativity. In theory, it could be as high as 6, the number of possible sites in a Rho hexamer, but this would be found only if the binding to the sites were fully cooperative and if each subunit had a site. Our evidence that a monomer can bind to an RNA by itself indicates that binding is not fully cooperative, and studies on the binding of RNA oligonucleotides indicate that hexameric Rho has three strong sites and three weak sites (*26*). Hence, the values of *n* between 1.5 and 2 are likely outcomes of only partial cooperativity and of the use of fewer than 6 high-affinity sites.

The B3′ RNA was chosen because it contains a natural *rut* element in an RNA that is similar in length to poly(rC) that is protected from RNase A digestion by Rho (*24*, *27*). It thus represents a good candidate for making a complex with RNA that would be suitable for structural studies. The finding that the apparent affinity of Rho for B3′ is about 4-fold lower than it is for the full-length *cro* RNA suggests that RNA outside of the *rut* region does contribute to the overall stability of the complex. Whether this extra stabilization is due to certain defined segments of the RNA or merely nonspecific electrostatic interactions with the larger RNA remains to be determined.

The different adenine nucleotides used, ATP, ADP and AMPPNP, all gave binding data that fit well to the monomeric binding curve. Since ADP had the same effect as ATP on stabilizing the oligomeric form of Rho (Figure 4), we conclude that the main influence of the different adenine nucleotides on the binding properties is related to their effect on the mode of binding and this effect is either the same or very similar regardless of whether the nucleotide is a substrate for hydrolysis. However, the apparent K_d values for the three adenine nucleotides were slightly different, showing higher affinity in the presence of ATP than with ADP or AMPPNP. This was true for the minimal *rut* B3′ RNA, which does not activate ATP hydrolysis very well (data not shown), and for the full-length *cro* RNA, which is a potent activator of ATP hydrolysis (*11*, *16*). Thus, there may be minor differences in the types of complexes formed with the different adenine nucleotides.

The presence of adenine nucleotides influences not only the mechanism of binding but also the stability of the complexes as well, as indicated by their effects on the apparent first-order dissociation rate constants. These have to be considered as apparent rate constants because dissociation, like binding, is likely a complex process involving loss of subunits rather than a unimolecular dissociation of hexameric Rho. The influence of ATP on complex stability is the probable reason for a discrepancy in reported dissociation rate values. Steinmetz and Platt (*25*) found that the *cro*−Rho complex dissociated with a half-life of 25 min,

whereas Martinez et al. (*28*) reported a value of about 4 min. The former measurement was made in a solution that contained ATP (in helicase reaction buffer) whereas the latter was made in a solution with similar ionic properties but that lacked ATP. Since ADP and AMPPNP have almost the same effects on stabilizing the complexes as ATP and since the stabilization with all the adenine nucleotides occurs as well for B3′ RNA as for the full-length *cro* RNA, we conclude that there is no special stabilization of Rho−RNA complexes that are actively undergoing ATP hydrolysis.

The complexes of Rho with B3′ RNA in the presence of ATP dissociate about 6-fold more slowly in 0.15 M potassium glutamate than in 0.05 M KCl (Table 2), yet the apparent equilibrium dissociation constant is 4-fold higher in the former solution than the latter (Table 1). Since an equilibrium dissociation constant is equal to the ratio of the dissociation rate constant over the association rate constant, this result indicates that the change from 0.05 M KCl to 0.15 M potassium glutamate reduces the rate constant for complex formation by more than a factor of 10. This could be related to the effects of ionic strength on the association state of free Rho because an increase in the ionic strength, whether from KCl (*12*) or potassium glutamate (*19*), favors dissociation of oligomeric Rho. However, once a complex is formed, its dissociation depends on the displacement power of the ions, which is known to be much higher for chloride ions than for glutamate ions. This result also suggests that the interaction with RNA does provide some further stabilization to the functional Rho hexamer.

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