The Kinetic Pathway of RNA Binding to the Escherichia coli Transcription Termination Factor Rho*

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The Escherichia coli transcription termination factor Rho is structurally and functionally homologous to hexameric helicases that assemble into ring structures. Using stopped-flow fluorescence and presteady-state ATPase kinetics, we have determined the kinetic pathway of poly(C) RNA binding to Rho hexamer, both in the presence and in absence of ATP. These studies indicate a four-step sequential mechanism of RNA binding and reveal the respective roles of the primary and secondary RNA binding sites in initiation and ATPase activation of Rho. The primary RNA binding sites of Rho hexamer interact with poly(C) RNA at a diffusion-limited rate constant close to 8 × 10^8 M^-1 s^-1, resulting in the Rho-RNA species PR1, which subsequently isomerizes to PR2 with a rate constant 21 s^-1. The PR2 isomerizes to PR3 with a rate constant of 32 s^-1 in the presence of ATP, and the formation of PR4 from PR3 results in a species that is fully competent in hydrolyzing ATP at the RNA-stimulated rate. The PR3 to PR4 isomerization occurs at a relatively slow rate of 4.1 s^-1; thus, the presteady-state ATPase kinetics show a distinct lag due to the slow initiation step. The interactions of the RNA with the primary sites trigger ring opening, and we propose that during the last two steps, the RNA migrates into the central channel and interacts with the secondary sites, resulting in the activation of the ATPase activity. The primary RNA binding sites, in addition to promoting sequence specific initiation, kinetically facilitate loading of the RNA into the secondary sites, which are relatively inaccessible, since they are present in the central channel. These studies reveal common features used by hexameric helicases to bind nucleic acids in an efficient and specific manner.

The Escherichia coli Rho protein is a transcription termination factor that assembles into a hexamer of six identical protein subunits arranged in a ring (1, 2). The hexamer is the functional form of the Rho protein, and its biological role is believed to translocate along the RNA in the 5′ to 3′ direction coupled to ATP hydrolysis. The translocation and/or unwinding activity of Rho results in the disruption of the RNA-DNA duplex in the elongation complex, which releases the transcript and causes transcription termination (6–8).

The interactions of the Rho hexamer with the RNA are mediated by two classes of RNA binding sites (9–12). The primary RNA binding site residing in the N-terminal domain of Rho polypeptide has affinity for pyrimidine-rich nucleic acid. The structure of the N-terminal domain of Rho protein complexed with oligo(rC)9 has been determined (13). These and other structural studies indicate that the primary RNA binding sites crown the Rho hexamer and are easily accessible for RNA binding (2, 13, 14). The RNA has been proposed to wrap around the primary RNA binding sites of the Rho hexamer (13, 16), consistent with the protection of 70–80 nucleotides of poly(C) RNA (15). Several studies indicate that the Rho hexamer contains a secondary RNA binding site that is distinct from the primary site (9, 11, 12, 17). Based on mutational and cross-linking studies, and the homology of Rho to the F1-ATPase, it has been proposed that the secondary RNA binding sites in the C-terminal domain reside within the central channel of hexamer ring (12). This mode of RNA binding is similar to the mode of DNA binding employed by several hexameric helicases such as T7 gp4, E. coli DnaB, and T4 gp41 (18–20). In Rho, the interactions of RNA with the secondary sites are necessary for ATPase activation (9).

The goals of the studies presented here were to determine the kinetic pathway of RNA binding to the Rho hexamer and to elucidate the roles of the primary and secondary binding sites in the initiation process. We have used stopped-flow method to monitor RNA binding in real time by following the RNA-induced changes in the intrinsic protein fluorescence of the Rho protein. The observed kinetics indicated a multistep mechanism for RNA binding. The intrinsic rate constant of each step was determined by globally fitting the kinetic data at various RNA concentrations to a four-step model using numerical methods. The four-step sequential mechanism consisted of a diffusion-limited bimolecular binding of poly(C) RNA to the Rho hexamer, which we propose represents interactions with the primary RNA-binding site located on the outer surface of the ring. The subsequent slower conformational changes represent ring opening and passage of the RNA strand into the central channel of the opened Rho ring. Because the ATPase rate is stimulated only when the RNA interacts with the secondary sites, we were able to determine the kinetics of RNA binding to the secondary sites by following the presteady-state kinetics of ATP hydrolysis. Conserved mechanisms of binding and initiation were revealed upon comparison of the mechanism of RNA binding of the Rho hexamer with the DNA binding mechanisms of hexameric helicases such as T7 gp4 (21) and E. coli DnaB (22).
Experimental Procedures

Protein, RNA Homopolymer, Nucleotide, and Buffers—Purified Rho protein was a gift from Dr. Katsuya Shigesada (Department of Genetics and Molecular Biology, Kyoto University, Kyoto, Japan). The Rho protein was overexpressed in E. coli strain HB101 carrying the Rho overexpression plasmid pKS26 (23) and purified according to Finger and Richardson (24) with slight modifications. The Rho protein concentration was determined by UV absorption at 289 nm using an extinction coefficient of 0.325 (mg/ml)⁻¹ cm⁻¹ (25). The ATP and RNA homopolymer, poly(C), were purchased from Amersham Pharmacia Biotech. Poly(C) RNA had a reported s₂₀,₀,₀ value of 7.1 in 0.15 M NaCl, 0.0015 M sodium citrate buffer, pH 7.0, with an average length of 420 bases. Poly(C) RNA concentration was determined by UV absorption at 289 nm using an extinction coefficient of 0.325 (mg/ml)⁻¹ cm⁻¹ for the cytosine base. The RNA was dissolved in TE buffer (40 mM Tris-HCl, pH 7.0, 0.5 mM EDTA) and used without further purification. [α-³²P]ATP was purchased from Amersham Pharmacia Biotech, and its purity was assessed by polyacrylamide-cellulose TLC and corrected for in all experiments. ATP was purchased from Sigma and used without further purification. Buffer B contains 40 mM Tris-HCl (pH 7.7), 100 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, and 10% (v/v) glycerol.

Fluorescence Measurements: Equilibrium RNA Binding—The fluorescence spectra (320–400 nm) of Rho protein with and without RNA or RNA plus ATP were measured at equilibrium with excitation at 290 nm in a Fluoromax-2 fluorometer (Instrumentation SA, Inc.) at constant temperature (18 °C). The fluorescence of Rho protein at 0.1 μM (hexamer) was measured in buffer B. Rho spontaneously assembles into a hexamer when present at 0.1 mg/ml. In the presence of RNA or ATP, Rho exists almost exclusively as a hexamer even at 1 μM (26). Hence, the concentration of Rho protein used in this study (0.1 μM hexamer) is well above the condition required to form the hexamer in the presence of poly(C) RNA. The emission spectrum was collected after polycy(C) RNA (0.3 μM) addition and after the addition of RNA and ATP (3 mM). The equilibrium fluorescence measurements were carried out within 5 min of the addition of RNA. During this time, the amount of ATP hydrolyzed is less than 1.0 mM (30 μM×hexamer ÷ 300 μM × 0.1 μM hexamer). The spectra were corrected for background fluorescence of buffer B and inner filter effects due to RNA and ATP absorbances at 290 nm using the following equation (27),
\[
F_{i,j} = F_{oe} \times 10^{\Delta A_{i,j}}
\]
(Eq. 1)

where \(F_{i,j}\) is the corrected fluorescence intensity at a particular wavelength; \(F_{oe}\) is the observed fluorescence intensity; \(A_{i,j}\) is the absorbance at 290 nm; and \(\Delta A_{i,j}\) is the absorbance at the emission wavelengths.

Stopped-flow Kinetics of RNA Binding—The stopped-flow kinetic experiments were performed using an instrument manufactured by KinTek Corp. (State College, PA). A 40-μl solution containing Rho (0.2 μM hexamer) with or without ATP (3 mM) in buffer B was rapidly mixed with a solution of poly(C) RNA (100–600 nM) at 18 °C in buffer B. The changes in the intrinsic fluorescence of Rho after mixing with the poly(C) RNA were monitored by exciting the sample at 290 nm and monitoring emission above 345 nm using a long pass filter (lp 345 filter from Oriel Corp.). Four to seven kinetic traces were averaged and fit to the sum of two exponentials (Equation 2) and to the appropriate RNA binding mechanisms (see below),
\[
F(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + C
\]
(Eq. 2)

where \(F(t)\) is the fluorescence intensity at time \(t\), \(C\) is a constant representing the fluorescence intensity at \(t = \infty\), \(A_1\) and \(A_2\) are the fluorescence amplitudes, and \(k_1\) and \(k_2\) are the observed rate constants. The 500 data points were distributed in two time windows to accurately resolve both the fast and slow phases. The observed rate constants of the fast and the slow phase were plotted against the poly(C) RNA concentration and fitted to either the linear equation or the hyperbolic equation (Equation 3),
\[
h_{obs} = \frac{V_{max}[RNA]}{K_{d} + [RNA]}
\]
(Eq. 3)

where \(h_{obs}\) is the observed rate at each [RNA], \(V_{max}\) is the maximum observed rate constant, and \(K_d\) is the concentration of RNA at which the observed rate is half of the maximum.

Presteady-state ATP Hydrolysis: Three-syringe Rapid Chemical Quenched-flow Experiments—The presteady-state kinetics of ATP hydrolysis were measured in a rapid chemical quenched-flow instrument built by KinTek Corp. In the three-syringe experiment mode, two delay times were used (KinTek RQF-3 software). An 8-μl solution of Rho protein (6.0 μM hexamer) in buffer B was rapidly mixed with an 8-μl mixture of ATP (2.0 mM) and [α-³²P]ATP (0.10 μCi/ml) for 5 or 10 s at 18 °C. After incubation for 5 or 10 s, the sample was mixed with 32 μl of poly(C) RNA (2.0 μM) in buffer B added from the third syringe of the quenched-flow instrument. The 48-μl reactions were incubated in the reaction loop for varying times (15 ms to 1.5 s) and mixed with 120 μl of 2.0 M formic acid to stop the reaction. Aliquots (1.0 μl) from each acid-quenched reaction at varying time points were spotted on polyethyleneimine-cellulose TLC, which was developed in 0.4 M potassium phosphate, pH 3.4. The resolved radioactive ATP and ADP were quantified in a PhosphorImager instrument (Molecular Dynamics, Inc., Sunnyvale, CA). Product formation was equal to the radioactivity corresponding to ADP divided by the total radioactivity. To estimate ATP hydrolysis in the first delay time, the acid quench was added from the third syringe instead of the RNA. This control experiment was repeated at least three times, and the values were averaged and taken as the background or hydrolysis at the zero time point. The efficiency of 2.0 M formic acid as a quenching reagent was also determined by including poly(C) RNA in the collecting microcentrifuge tube under the same setup as performed in the zero time point control. Less than 0.08% of ATP was hydrolyzed to ADP in both control experiments, which was the same as the level observed in the absence of enzyme.

Quantitative Analysis and Global Fitting of the Presteady-state Kinetic Data—The intrinsic rate constants were determined by global fitting of the stopped-flow kinetic data collected at increasing concentrations of poly(C) RNA. The global nonlinear least-squares fitting was performed using the software “Scientist” (MicroMath Research, SLC, UT). After choosing a model for RNA binding, differential equations were written for each kinetic species in the mechanism. For global fitting, a separate set of differential equations, distinguished by different suffixes, were written for each RNA concentrations. The stopped-flow fluorescence traces were directly fitted by assigning the observed fluorescence at any given time, \(F(t)\) as the sum of the background fluorescence (\(F_B\)), which is the free protein fluorescence, and the fluorescence of each Rho-RNA species (\(PR_i\)) in the mechanism.

\[
F(t) = \sum_{i=1}^{N} F_i + PR_i(t) + F_B
\]
(Eq. 4)

where \(F_i\) is the specific fluorescence of each Rho-RNA species, and \(F_B\) is the background fluorescence due to buffer and free protein. \(PR_i\) is the amount of each Rho-RNA species at any given time, which changes during the time course of RNA binding. The initial estimates for the rate constants during global fitting of the kinetic data were obtained from the quantitative analysis of the observed rate constants versus RNA concentrations. The process of global fitting involved first, fixing the rate constants and determining the specific fluorescence values (\(F_i\)). Subsequently, one or the other set of parameters was kept constant, and global fitting was used to optimize the floating set. Eventually, all the parameters were floated to fit all the data sets to a single mechanism. The fitting process was governed by a modified Marquard-Levenberg algorithm making use of the analytical Jacobian matrix. The quality of the fit was judged by visual inspection of overlays of the fitted curves and the data as well as inspection of the residuals.

The presteady-state ATP hydrolysis kinetic data were fit to the RNA binding mechanism in the presence of ATP. Each of the Rho-RNA species (\(PR_i\)) was assigned an ATP hydrolysis rate. To fit the burst of three ATPs/hexamer, an exponential term was included where \(PR_1\) species was hydrolyzing ATP. The data fit best when \(PR_1\) hydrolyzed three ATPs at the rate of \(k_1 = 163 \; s^{-1}\); \(PR_4\) hydrolyzed ATP at the RNA-stimulated rate, \(k_{stim} = 30 \; s^{-1}\); and the rest of the species (\(PR_2\) and \(PR_3\)) were hydrolyzing ATP at the unstimulated rate, \(k_5 = 3.0 \times 10^{-4} \; s^{-1}\). Thus, the formation of ADP at any given time was described by:

\[
dH/dt = 3 \times PR_1 k_1 \exp(-h_1 t) + k_5 (PR_2 + PR_3) + h_{stim} PR_4
\]
(Eq. 5)

The presteady-state ATP hydrolysis data provided an additional constraint to be satisfied by the RNA binding mechanism in the presence of ATP. Therefore, the resulting intrinsic rate constants in the RNA binding mechanism in the presence of ATP (see Table I) were obtained by fitting globally stopped-flow as well as the acid quenched-flow ATP hydrolysis data.
RESULTS

We have investigated the transient state kinetics of RNA binding to *E. coli* Rho hexamer with the goal of elucidating both the mechanism of initiation and the roles of the primary and secondary binding sites in RNA binding. We used poly(C) RNA as the ligand because it is an established substrate that binds to the Rho hexamer with a high affinity (K_d of 1.0 nM) and stimulates the Rho ATPase nearly 10^5-fold (15, 28). To follow the kinetic pathway of RNA binding in real time, we monitored the RNA-induced changes in the intrinsic fluorescence of Rho protein using the stopped-flow method. To determine when the Rho-RNA species becomes competent in ATPase activity, we showed that a stable Rho-RNA species whose fluorescence was greater than free Rho protein was formed after the bimolecular event. The rate constant for the bimolecular binding event was estimated from the slope that was equal to 7.5 × 10^8 M^-1 s^-1. The rate constant of the second phase (k_2) increased in a hyperbolic manner with [RNA] and reached a plateau at high poly(C) RNA concentration (Fig. 2D). The hyperbolic dependence provided evidence for a conformational change in the Rho-RNA complex following the bimolecular encounter. The saturation at about 4.7 s^-1 provided an estimate for the rate constant of this conformational change. Thus, the fluorescence stopped-flow studies indicated a minimal two-step mechanism for RNA binding consisting of a bimolecular association between Rho and poly(C) RNA followed by an isomerization step.

We next attempted to globally fit the kinetic data at various RNA concentrations to a two-step sequential mechanism using the Scientist program that uses numerical methods to solve the differential equations that describe the mechanism. The global fitting procedure is described briefly under “Experimental Procedures” and in more detail elsewhere (29). We were not able to obtain a global fit with the two-step mechanism. Therefore, a three-step mechanism was used as the model, which provided a good fit with the intrinsic rate constants shown in Table I. This mechanism showed that the Rho hexamer interacts with the RNA polymer to form PR1 at a diffusion-limited rate constant k_1 = 7.5 × 10^8 M^-1 s^-1, and the complex dissociates with a rate constant of 12.0 s^-1. The collision complex PR1 isomerizes to PR2 at a forward rate of k_2 = 26.2 s^-1 and a reverse rate of k_-2 = 2.8 s^-1. The PR2 subsequently converts to PR3 at a relatively slow rate, k_3 = 5.0 s^-1. There was no detectable rate (k_-3) for the conversion of PR3 to PR2.

Stopped-flow Kinetics of Poly(C) Binding to Rho Hexamer in the Presence of ATP—Previous studies have shown that ATP increases the affinity of Rho protein for RNA (30). Under equilibrium conditions, in the absence of RNA, the Rho hexamer binds three or four ATP molecules with very little hydrolysis (31). The kinetics of RNA binding to Rho-ATP complex were therefore measured by mixing poly(C) RNA (0.1 μM) with Rho protein (0.1 μM hexamer, final concentration) containing 3.0 mM ATP in buffer B, in a stopped-flow instrument. The fluorescence changes in the Rho protein upon RNA binding were monitored in the rapid time scale and shown in Fig. 3A. The initial interactions of the Rho hexamer with RNA led to an increase in fluorescence at a rapid rate, and a subsequent

![Image](https://example.com/image.png)
conformational change decreased Rho protein fluorescence. The increase and decrease in protein fluorescence with time were fit to the sum of two exponentials with rate constants, 118 s$^{-1}$ and 17.4 s$^{-1}$, respectively (Fig. 3A). Note that the observed rate constants of the fast phases are almost the same, with or without ATP, but the rate constant of the second phase with ATP is about 4 times faster.

To determine the mechanism of RNA binding in the presence of ATP, the fluorescence stopped-flow studies were carried out at constant Rho and varying poly(C) RNA concentrations. The final concentrations of the Rho hexamer and poly(C) RNA were 0.1 μM and 50–200 nM, respectively. The kinetic data were collected up to 1.0 s, and during this period the amount of ATP hydrolyzed was less than 10 μM. Hence, the concentration of ATP substrate did not change during the course of our measurements. The kinetic data (Fig. 3B) were fit to the sum of two exponentials and globally fit to a kinetic model using numerical approaches. The rate of the fast phase increased linearly with increasing [RNA] with a slope of 8.5 × 10$^6$ M$^{-1}$ s$^{-1}$ and an intercept of 11.0 s$^{-1}$. The slower rate constant ($k_2$) increased hyperbolically with RNA concentration and fit to a hyperbola (Equation 3) with a $K_m$ of 50 nM, a maximum rate of 4.7 ± 0.6 s$^{-1}$, and a y intercept of 1.2 s$^{-1}$.

The RNA binding kinetics with ATP at varying [RNA] also fit a two-step model. The global fit was carried out as described above using the program Scientist. The solid black lines in Fig. 3B are the resulting fits to the three-step RNA binding mechanism, shown in Table I. The derived intrinsic rate constants indicate that the initial bimolecular association of Rho-ATP complex and RNA result in PR1 with $k_1$ of 8.5 × 10$^6$ M$^{-1}$ s$^{-1}$ and $k_{-1}$ of 22.9 s$^{-1}$. The conversion of PR1 to PR2 occurs at a rate constant, $k_2$, of 20.5 s$^{-1}$ and a reverse rate constant, $k_{-2}$, of 1.6 s$^{-1}$, which is close to the $k_2$ and $k_{-2}$ in the absence of ATP. The PR2 is converted to PR3 at a rate constant, $k_3$, of 31.2 s$^{-1}$, which is 6 times faster than the rate of the corresponding step in the absence of ATP. Therefore, ATP does not affect the first and second steps of RNA binding, but ATP does accelerate the third step.

The Presteady-state Kinetics of ATP Hydrolysis—The above stopped-flow fluorescence studies show that at least three Rho RNA species, PR1, PR2, and PR3, are formed during the process of RNA binding to the Rho hexamer, and the global analysis provides the rate constants of each step. To fully understand the mechanism of RNA binding, one however needs to know the structures of the Rho-RNA complexes that accumulate during the reaction. Unfortunately, at present, the protein fluorescence changes cannot be interpreted in terms of the structures of the complexes, and other studies are necessary. Several studies have shown that Rho contains two classes of nucleic acid binding sites, the primary and the secondary RNA-binding sites (11, 32). The primary RNA binding site is accessible, whereas the secondary site is proposed to lie within the central channel (12). It is also known that the steady-state ATPase activity of Rho is stimulated only when RNA interacts with the secondary RNA-binding sites (9). Thus, RNA needs to travel
Transient State Kinetics of RNA Binding to E. coli Rho

### Table I

| Three-step mechanism of poly(C) RNA binding to E. coli Rho in the absence or presence of ATP |
|---|---|---|---|---|---|---|
| $k_1$ | $k_{-1}$ | $k_2$ | $k_{-2}$ | $k_3$ | $k_{-3}$ |
| Without ATP | $7.47 \pm 0.14 \times 10^6$ | $12.0 \pm 0.77$ | $26.2 \pm 1.53$ | $2.81 \pm 0.86$ | $5.00 \pm 0.46$ | $<0.02$ |
| With ATP | $8.51 \pm 0.13 \times 10^6$ | $22.9 \pm 1.23$ | $20.5 \pm 0.77$ | $1.58 \pm 1.51$ | $31.2 \pm 1.93$ | $0.025 \pm 0.013$ |

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Fig. 3. Stopped-flow kinetics of Rho protein interaction with poly(C) RNA in the presence of ATP. A, the Rho protein (0.1 μM hexamer, final concentration) and ATP (3 mM) in buffer B were mixed with poly(C) RNA (0.10 μM) at 18°C in a stopped-flow instrument, and the fluorescence of Rho was measured after mixing upon excitation at 290 nm. The resulting kinetics showed two phases, and the residuals show best fit to the sum of two exponentials (Equation 2) with the residuals show best fit to the sum of two exponentials (Equation 2).

The burst of ATP hydrolysis coincided with the formation of PR1. This suggests that the initial encounter of poly(C) RNA with the Rho-ATP complex results in the hydrolysis of three ATP molecules. The subsequent lag in ADP formation indicates that the PR1 species is not competent in catalyzing multiple ATPase turnovers at the RNA-stimulated steady-state rate. The kinetic lag indicates that the formation of the Rho-RNA species that is competent in catalyzing multiple turnovers of ATP hydrolysis occurs at a slower rate.

The next task was to determine which of the Rho-RNA species, PR2 and/or PR3, were ATPase-competent. We used the three-step sequential mechanism of RNA binding and the rate constants derived from the stopped-flow studies to simulate the ATPase kinetics, as described under “Experimental Procedures.” In the model where PR1, PR2, and PR3 were all capable of hydrolyzing ATP, no lag kinetics were predicted. When PR2 and PR3 or when PR3 alone was ATPase-competent, then the predicted lag was too short, as shown by the “dashed line” in Fig. 4B. These kinetic simulations indicated that PR3 was not competent in hydrolyzing ATP and needed to be converted to PR4 to hydrolyze ATP at the RNA-stimulated rate. Thus, a PR3 to PR4 isomerization step was added to the three-step mecha-
The presteady-state kinetics of ATP hydrolysis. A, the diagram shows the design of the three-syringe acid-quenched experiment. The Rho protein (3.0 μM hexamer) was mixed with [α-32P]ATP and Mg-ATP (1.0 mM) for 5 or 10 s (t1) in buffer B at 18 °C. Subsequently, the Rho-ATP complex was mixed with the poly(C) RNA (1.34 μM polymer), and the reaction was quenched after varying times (t2). B, the presteady-state ATP hydrolysis kinetic experiments were repeated five times, and the average data are shown. The solid line is the fit to the four-step RNA binding mechanism, shown in Table II. In this mechanism, three ATPs per hexamer were initially hydrolyzed by PR1 at a fast rate of 163 s⁻¹, and only PR4 was capable of hydrolyzing ATP at the RNA-stimulated rate of 30 s⁻¹. The dashed line shows the predicted curve for a mechanism in which PR3 and PR4 hydrolyzed ATP at the poly(C) RNA-stimulated rate of 30 s⁻¹.

**DISCUSSION**

The Rho protein self-assembles into a hexamer, with or without ATP, and catalyzes sequence-specific transcription termination in vivo. This process requires initiation or binding of the Rho hexamer to a specific site on the RNA, followed by unidirectional translocation along the RNA and disruption of the RNA/DNA hybrid in the elongating complex. Very little is known about the kinetics of initiation and the mechanism by which RNA binds to the Rho hexamer. There is ample evidence that both the primary and secondary RNA binding sites mediate the interactions of the Rho hexamer with the RNA, but the dynamics of the interactions have not been elucidated. The Rho protein is homologous to the hexameric helicases, and thus comparative studies of Rho with other hexameric helicases will also provide general mechanisms that these ringed proteins utilize in binding and translocating along the nucleic acids.

We have used presteady-state kinetics to elucidate the dynamics of Rho protein interaction with poly(C) RNA. The kinetic pathway was determined by monitoring the intrinsic fluorescence changes in the Rho protein upon RNA binding using the stopped-flow method and by measuring the presteady-state ATP hydrolysis activity. The fluorescence of Rho protein increases when it binds to poly(C) RNA in the absence of ATP, and the fluorescence decreases when it binds to RNA in the presence of ATP. These fluorescence changes most likely result from global conformational changes that affect the quantum yield of the tryptophan or both the tryptophan and the tyrosine residues in the Rho protein, and they provide the necessary signals to monitor in real time the kinetics of RNA binding.

The transient changes in the fluorescence of the Rho protein upon RNA binding were measured by the stopped-flow method, and the resulting kinetics at varying concentrations of RNA were fit to a kinetic model using the program Scientific. Both global fits of the fluorescence stopped-flow kinetic data, with and without ATP, were obtained to a three-step sequential mechanism, shown in Table I. The presteady-state ATPase kinetics however indicated the presence of a fourth slower step in the mechanism. This last step was necessary for the formation of a Rho-RNA species that was competent in catalyzing ATPase turnover at the RNA-stimulated level. The four-step mechanism shown in Table II is therefore consistent with both
the Rho hexamer and poly(C) RNA were 100 and 150 nM, respectively.

Four-step RNA binding mechanism in Table II. The concentrations of various species in the RNA binding pathway were simulated using the hexamer in the presence of ATP.

A

The fluorescence stopped-flow and the presteady-state ATPase kinetics. The four-step sequential mechanism illustrated in Fig. 6 indicates that the poly(C) RNA binds to the Rho hexamer, both with and without ATP, at a diffusion-limited rate ($k_1 = 8.6 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$) and the resulting complex PR1 dissociates with a rate, $k_{-1} = 23.2 \text{ s}^{-1}$. The PR1 complex has a dissociation constant of about 30 nM, but it is a transient species that isomerizes to PR2 with a forward rate of $k_{2} = 20.8 \text{ s}^{-1}$ and a reverse rate of $k_{-2} = 1.60 \text{ s}^{-1}$. The nucleotide, ATP, does not play a significant role during the formation of PR1 and PR2. The conversion of PR2 to PR3 is, however, stimulated by ATP. In the presence of ATP, the PR2 intermediate isomerizes to PR3 with a forward rate, $k_{3}$, of 31.6 s$^{-1}$ and reverse rate, $k_{-3}$, of 1.02 s$^{-1}$. In the absence of ATP, the PR2 is converted to PR3 at a rate of 5.0 s$^{-1}$. The PR3 then appears to irreversibly convert to PR4 at a rate, $k_{4}$, of 4.1 s$^{-1}$ in the presence of ATP. The presteady-state ATPase kinetics indicated that PR4 is the only species that is capable of hydrolyzing ATP at the RNA-stimulated level.

The presteady-state ATPase kinetics showed a burst of ATP hydrolysis, in which three ATPs were rapidly hydrolyzed per Rho hexamer, after adding RNA. Presumably, we start with a Rho-ATP complex, with three or four ATPs bound per hexamer (31, 33), and it appears that Rho hydrolyzes three ATPs rapidly upon its initial encounter with the RNA. A similar experiment in the literature (34) done under chase conditions (where RNA was added along with excess nonradioactive ATP that acts as a chase) showed hydrolysis of three ATPs as well, consistent with the above proposal. The kinetic lag following the burst of ATP hydrolysis indicates that PR1 is not capable of turning over ATP. The kinetic simulations indicated that the species from PR1 to PR3 were incapable of hydrolyzing ATP at the RNA-stimulated rate. The presteady-state ATPase kinetics provided evidence for a fourth step in the RNA binding process, PR3 to PR4 conversion, at a relatively slow rate close to 4 s$^{-1}$. The kinetics indicate that PR4 is the only species that is competent in carrying out multiple ATP hydrolysis events. The hydrolysis of three ATPs at a rapid rate therefore may be a peculiarity of the initiation process. If ATP hydrolysis is coupled to translocation, then this study suggests that PR4 is the only species capable of translocating along RNA.

Previous studies have shown that the Rho hexamer contains two classes of nucleic acid binding sites, the primary and secondary binding sites (10, 11). The primary site binds pyrimidine-rich RNA or DNA with a high affinity (13). The atomic structure of the N-terminal RNA-binding domain consisting of the primary binding sites has been solved, and it was suggested that the primary sites crown the hexamer (13, 14). Electron microscopy studies of the Rho hexamer in the presence of tRNA showed that it binds to the outer periphery and probably to the primary sites. Upon binding to the tRNA, the Rho hexamer showed significant deviation from the 6-fold symmetry, with “notched” or open rings (2). These studies suggest that the primary RNA binding sites face outward in the hexameric ring, and when the RNA binds to these sites, the open form of the Rho hexamer is stabilized. On the other hand, the secondary sites have been shown to bind nucleic acids weakly, and RNA binding to these sites is allosterically coupled to ATP binding and hydrolysis (9, 11). The secondary sites also prefer C-rich RNA (15), and several studies indicate that these sites lie within the central channel of the Rho ring (12, 17).

The fluorescence stopped-flow and the presteady-state ATPase kinetics have provided a sequential RNA binding mechanism that is illustrated in Fig. 6. The rate constants were accurately determined by global fitting, and available results have been considered in proposing the structures of the intermediate complexes (2, 16, 32), including the “tethered-tracking” model (8). Our model (Fig. 6) involves the following steps:

$\text{P + R} \rightarrow \text{PR1} \rightarrow \text{PR2} \rightarrow \text{PR3} \rightarrow \text{PR4}$

$$
\begin{array}{cccccccc}
     & k_1 & k_2 & k_3 & k_4 \\
PR & k_{-1} & k_{-2} & k_{-3} & k_{-4} \\
PR1 & 8.6 \pm 0.67 \times 10^8 & 23.2 \pm 4.65 & 20.8 \pm 2.62 & 1.60 \pm 0.74 & 31.6 \pm 4.6 & 1.02 \pm 0.32 & 4.10 \pm 0.59 \\
PR2 & 0.32 \pm 4.10 & 0.59 \pm 1.02 & 3.2 \pm 1.02 & 0.74 \pm 31.6 & 0.59 \pm 1.02 & 23.2 \pm 4.65 & 20.8 \pm 2.62 \\
PR3 & 0.74 \pm 31.6 & 0.59 \pm 1.02 & 23.2 \pm 4.65 & 20.8 \pm 2.62 & 1.60 \pm 0.74 & 31.6 \pm 4.6 & 1.02 \pm 0.32 \\
PR4 & 1.02 \pm 0.32 & 4.10 \pm 0.59 & 20.8 \pm 2.62 & 1.60 \pm 0.74 & 31.6 \pm 4.6 & 1.02 \pm 0.32 & 4.10 \pm 0.59 \\
\end{array}
$$

**TABLE II**

Four-step mechanism of poly(C) RNA binding to E. coli Rho hexamer in the presence of ATP

**FIG. 5.** Kinetic simulation of poly(C) RNA binding to the Rho hexamer in the presence of ATP. A, the formation and decay of the various species in the RNA binding pathway were simulated using the four-step RNA binding mechanism in Table II. The concentrations of the Rho hexamer and poly(C) RNA were 100 and 150 nM, respectively. The open circles show the decay of free protein (P) as it binds to the RNA. The triangles, squares, and diamonds show the formation and decay of the intermediates PR1, PR2, and PR3, respectively. The filled circles show the formation of the final, stably bound Rho-RNA complex (PR4), which is competent for ATP hydrolysis at a rate of $k_{cat}$. B, relative protein fluorescence intensities of each kinetically distinguishable species are shown by bar graphs. The total fluorescence of PR1 was set to 100%.

- **A**
  - Protein species, nM
  - Time, sec
  - Protein species:
    - P
    - PR1
    - PR2
    - PR3
    - PR4
  - Fluorescence
  - Relative Fluorescence (%)

- **B**
  - Rho-RNA Species
  - Relative Fluorescence (%)
(a) RNA binding on the outside of the ring; (b) the wrapping of the RNA in the continuous clefts of the primary RNA-binding sites that crown the hexamer ring; (c) the passage of the RNA into the central channel of the ring by the formation of a “notch” within the six-membered ring; and (d) the activation of the coupled ATPase-translocation (5′ → 3′) within the central channel.

The initial encounter of the RNA with the Rho hexamer occurs at a diffusion-limited rate constant, and we have observed that this binding is independent of ATP. Because the secondary RNA binding sites in the central channel would not be easily accessible, we propose that the initial diffusion-limited encounter of the RNA take place with few of the primary RNA-binding sites resulting in PR1. Because the formation of PR2 is also independent of ATP, we suggest that the RNA wraps around the continuous RNA binding clefts that crown the hexamer ring during the formation of PR2. In the presence of ATP, the PR2 isomerizes to PR3 at a relatively fast rate, and we propose that during this step the RNA migrates into the central channel and interacts with the secondary RNA binding sites. It is very likely that the RNA retains its interaction with the primary site even after it contacts the secondary site (as proposed by the tethered-tracking model). The last step may represent the closing of the ring around the RNA resulting in PR4, which is the species competent in turning over ATP, coupled to translocation.

The proposed ring-opening mechanism is consistent with the model in which the 3′-end of the RNA from the initial attachment site passes through the central channel in the hexamer ring (16). One possible mechanism that does not require the ring opening would be the threading model. In the threading mechanism, none of the subunit interfaces would be disrupted, and a free end of the RNA would thread into the hole. However, this mechanism is unlikely because Rho does not bind very tightly to an RNA lacking the rut site (35), and threading would be obstructed by the presence of the translating ribosomes on the nascent mRNA. Hence, the ring opening is likely to be a preferred pathway for loading hexameric Rho on the RNA under cellular conditions.

A general problem for ring proteins that bind nucleic acids in the central channel is coordinating the events of ring opening and nucleic acid binding in the central channel. In Rho protein, the RNA first binds to the primary site, which we propose acts as a Rho-loading site, and the prebound RNA is then transferred into the central channel upon ring opening. Such a model provides a mechanism for both sequence-specific loading of Rho on the RNA and for increasing the efficiency of RNA binding in the central channel. This mechanism appears to be general in the hexameric helicases that form a ring. The bacteriophage T7 gp4 has been proposed to use the primase site as a loading site to allow efficient binding to ssDNA (36). Consistent with this hypothesis, the hexameric *E. coli* DnaB, which does not contain a loading site within its polypeptide, binds single-stranded DNA nearly 1000-fold more slowly than the rate observed for Rho and T7 gp4 (22). The DnaB in fact interacts with accessory proteins, such as the DnaC protein, which loads DnaB at the replication origin (37). It is therefore very likely that DnaC will kinetically facilitate the loading of DnaB on the DNA. The hexameric helicases such as the SV40 large T antigen and BPV E1 have distinct origin-binding sites that may be the loading sites for binding at the specific origins of replication (38, 39). Phage T4 gp41 helicase associates with gp59 to load onto T4 gp32-coated DNA (40). The MCM helicases from eukaryotes and archaea interact with proteins such as Cdc6 that help load the helicase at the replication origin (41, 42). The various accessory proteins therefore act as loading sites for helicases (21). The hexameric helicases appear to use a general mechanism for nucleic acid binding. The helicases form a preassociation complex that brings the DNA or RNA in the vicinity of the central channel and increases the efficiency and specificity of loading the nucleic acid into the central channel.

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