Nucleotide Binding Induces Conformational Changes in *Escherichia coli* Transcription Termination Factor Rho*

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E. coli Rho protein contains two types of nucleic acid binding sites, referred to as the primary and the secondary sites (9). The primary nucleic acid binding sites reside in the N-terminal domains that form a crown over the Rho hexamer (10), and these sites bind pyrimidine-rich DNA or RNA with a high affinity (11). In addition to wrapping around the primary sites, the RNA but not the DNA interacts with the secondary sites in the central channel of the hexamer (12). The kinetics of RNA binding to the Rho hexamer showed that the initial binding of RNA occurs at a diffusion-limited rate constant to the primary sites (13). The primary sites act as loading sites that facilitate the binding or RNA loading into the central channel. Poly(C) RNA stimulates the Rho ATPase activity nearly 10^5-fold, and it needs to contact both the primary and the secondary sites for ATPase stimulation (9, 14).

A recent crystallography analysis shows an open ring structure of the Rho hexamer that resembles a lock washer (10), similar to the structures observed by electron microscopy (15). In the crystal structure, the RNA or the DNA is wrapped around the primary sites, but the RNA is not contacting the secondary sites. Although AMPPPNP nucleotide is bound to each subunit interface, the interfaces are open and not fully organized to catalyze the hydrolysis of ATP, and Mg(II) is not evident. It was proposed that this structure of Rho represents a state that is poised to load the RNA into the central channel, and that when RNA contacts the secondary sites in the central channel, it will lead to ring closure and the stimulation of ATP hydrolysis.

We have measured the kinetics of nucleotide binding utilizing ATP, fluorescent ATP, and unhydrolyzable ATP analogs to characterize the conformational changes in the Rho protein upon nucleotide and nucleic acid binding. Our results show that the structure of RNA-free Rho is distinct from the RNA- or DNA-bound Rho hexamer. The kinetics of nucleotide binding and dissociation show that the ATP-binding sites are not readily accessible, indicating that the subunit interfaces are closed in the absence of RNA. In the presence of RNA or DNA, the association and dissociation of nucleotides from Rho become fast, indicating that the subunit interfaces are open when RNA or DNA is bound to the primary sites. With the Rho-RNA complex but not the DNA complex, the fast bimolecular binding of ATP is followed by a protein conformational change at a rate very close to the rate of ATP hydrolysis. Based on the observed kinetics of nucleotide binding, we propose that the protein conformational change after ATP binding represents RNA binding to the secondary sites and possibly ring closure that is poised to unwind RNA/DNA heteroduplexes (1–5). Although the exact mechanism by which Rho causes transcription termination is not known, Rho protein binds nascent mRNA at specific loading sites, and it is believed that Rho translocates along RNA until it reaches the transcription complex, where it disrupts the transcription ternary complex (3, 6). Translocation along nucleic acid is, therefore, a basic activity of Rho similar to helicases (7, 8), and understanding it requires the knowledge of how nucleotide binding and hydrolysis events at the multiple nucleotide-binding sites on the Rho hexamer are coordinated to the mechanics of protein translocation. In this study, we measured the kinetics of nucleotide binding to Rho complexed to RNA or DNA that reveal conformational changes in the Rho protein that we believe are important for RNA binding and ATP hydrolysis.

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**The Escherichia coli** transcription termination factor Rho is a hexameric protein that has the ability to unwind RNA/DNA heteroduplexes (1–5). Although the exact mechanism by which Rho causes transcription termination is not known, Rho protein binds nascent mRNA at specific loading sites, and it is believed that Rho translocates along RNA until it reaches the transcription complex, where it disrupts the transcription ternary complex (3, 6). Translocation along nucleic acid is, therefore, a basic activity of Rho similar to helicases (7, 8), and understanding it requires the knowledge of how nucleotide binding and hydrolysis events at the multiple nucleotide-binding sites on the Rho hexamer are coordinated to the mechanics of protein translocation. In this study, we measured the kinetics of nucleotide binding to Rho complexed to RNA or DNA that reveal conformational changes in the Rho protein that we believe are important for RNA binding and ATP hydrolysis.

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**References**

1. The abbreviations used are: AMPPNP, 5'-adenylyl-β,γ-imidodiphosphate; FRET, fluorescence resonance energy transfer; mant-ATP, 2'-O-(N-methylanthraniloyl) adenosine-5'-triphosphate; ATPγS, adenosine-5'-O-(thiotriphosphate).
results in a Rho hexamer that is competent in nucleotide hydrolysis and translocation.

MATERIALS AND METHODS

Protein, RNA, and Buffer—Rho protein was over-expressed in E. coli strain HB101 transformed with pSK26R (rho; amp) and pNT203 (λCh57AN, tet') that was kindly provided by Dr. Katsumi Shigesada (Department of Genetics and Molecular Biology, Kyoto University, Kyoto, Japan) (16). The plasmid DNA was sequenced, and the wild-type Rho sequence was confirmed. The cells were grown at 32 °C, and Rho was heat-induced at 42 °C and purified according to the procedure of Finger and Richardson (17), with slight modifications. Rho protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of 0.325 (mg/ml)^{-1} cm^{-1} for the cytosine base. RNA was dissolved in TE buffer (40 mM Tris-HCl, pH 7.7, 100 mM KCl, 10 mM MgCl_2, 0.1 mM dithiothreitol, and 10% (v/v) glycerol. Poly(C) RNA had a reported extinction coefficient of 6200 M^{-1} cm^{-1} for the cytosine base. RNA was dissolved in TE buffer (40 mM Tris-HCl, pH 7.0, 0.5 mM EDTA) and used without further purification. dATP was a kind gift from Dr. Susan Gilbert (University of Pittsburgh). [34S]-Methylisatoic anhydride purchased from Molecular Probes (Eugene, OR) was used to fluorescently label the adenine nucleotide. Tris-HCl buffer contains 40 mM Tris-HCl, pH 7.0, 0.5 mM EDTA and 0.1 mM MgCl_2. The extinction coefficient of 0.325 (mg/ml)^{-1} cm^{-1} for the cytosine base was used for the calculations.

Nucleotides and Nucleotide Analogues—ATP, AMP-PNP, and ATP-S were purchased from Sigma and used without further purification. 3'-Mant-2'-dATP was purchased from Jena Bioscience, and 2'-methyl-3'-dATP was a kind gift from Dr. Susan Gilchrist (University of Pittsburgh). 3'-Mant-2'-dATP was synthesized and characterized as described (20). N-Methylisatoic anhydride purchased from Mofar Probes (East Brunswick, NJ) was used to fluorescently label the adenine nucleotide. The resulting products were fractionated using a DEAE-Sephacel column with a linear gradient of 20 mM to 1.0 mM triethylammonium bicarbonate buffer (pH 7.6). The nucleotides were resolved by thin-layer chromatography on silica gel 60, F254 (Aldrich Chemicals) in 1-propanol/ NH_4OH/water 6:3:1 (v/v), containing 0.5 g/liter EDTA. The nucleotide fluorescence amplitudes, and fluorescence change was small, and thus many traces were averaged to obtain the ATP binding kinetics. The observed rate of the protein fluorescence change increased linearly with [ATP] (Fig. 1B), and the slope (0.035 ± 0.0016 μM^{-1} s^{-1}) provided an estimate of the ATP binding rate, and the y-intercept (0.2 s^{-1}) provided an estimate of the ATP dissociation rate. Similar experiments with the unhydrolyzable ATP analog, AMP-PNP, provided a binding rate of 0.045 ± 0.002 μM^{-1} s^{-1} similar to the ATP-binding rate (Table I).

Kinetics of Nucleotide Binding to Rho—The kinetics of nucleotide binding were measured by following the changes in the intrinsic fluorescence of Rho after it was rapidly mixed with the nucleotide in a stopped-flow instrument. Each subunit of Rho hexamer contains one tryptophan close to the nucleotide-binding site at the subunit interface (10). When Rho was mixed with ATP in a stopped-flow instrument, a time-dependent decrease in protein fluorescence was observed (Fig. 1A). The protein fluorescence change was small, and thus many traces were averaged to obtain the ATP binding kinetics. The observed rate of the protein fluorescence change increased linearly with [ATP] (Fig. 1B), and the slope (0.035 ± 0.0016 μM^{-1} s^{-1}) provided an estimate of the ATP binding rate, and the y-intercept (0.2 s^{-1}) provided an estimate of the ATP dissociation rate. Similar experiments with the unhydrolyzable ATP analog, AMP-PNP, provided a binding rate of 0.045 ± 0.002 μM^{-1} s^{-1} similar to the ATP-binding rate (Table I).

RESULTS

Kinetics of Nucleotide Binding to Rho—The kinetics of nucleotide binding were measured by following the changes in the intrinsic fluorescence of Rho after it was rapidly mixed with the nucleotide in a stopped-flow instrument. Each subunit of Rho hexamer contains one tryptophan close to the nucleotide-binding site at the subunit interface (10). When Rho was mixed with ATP in a stopped-flow instrument, a time-dependent decrease in protein fluorescence was observed (Fig. 1A). The protein fluorescence change was small, and thus many traces were averaged to obtain the ATP binding kinetics. The observed rate of the protein fluorescence change increased linearly with [ATP] (Fig. 1B), and the slope (0.035 ± 0.0016 μM^{-1} s^{-1}) provided an estimate of the ATP binding rate, and the y-intercept (0.2 s^{-1}) provided an estimate of the ATP dissociation rate. Similar experiments with the unhydrolyzable ATP analog, AMP-PNP, provided a binding rate of 0.045 ± 0.002 μM^{-1} s^{-1} similar to the ATP-binding rate (Table I).

Kinetics of Nucleotide Binding to Rhopoly(C)—Next, we measured the kinetics of ATP binding to the Rhopoly(C) complex. Biphasic kinetics were observed upon mixing ATP with a complex of Rho and poly(C) RNA. An initial time-dependent increase in protein fluorescence was followed by a decrease in fluorescence (Fig. 2A). The fluorescence enhancement rate increased linearly with [ATP], with a slope of 2.5 ± 0.1 μM^{-1} s^{-1} and a y-intercept of 7.8 ± 2.5 s^{-1} (Fig. 2C). The fluorescence-quenching rate increased hyperbolically with increasing [ATP], reaching a plateau value (k_{max}) of 47 ± 6 s^{-1} with a K_c = 23 ± 1.4 μM (Fig. 2D). Because the rate of each phase depends upon [ATP], both phases are coupled to the ATP-binding step. The fast phase measures the bimolecular ATP-binding step, because the rate increases linearly with [ATP]. Comparison of the ATP-binding rate with and without RNA shows that the Rho-RNA binds ATP about 70-times faster than the RNA-free Rho. Thus, the ATP-binding sites are more accessible when RNA is bound to the Rho hexamer. The second phase, which was observed only with the Rho-RNA complex upon ATP-binding, must correspond to a protein conformational change, because the rate increases hyperbolically with [ATP] (23). Because the kinetic parameters (k_{max} and K_c) of the second phase
are close to the ATPase $k_{cat}$ (35–40 s$^{-1}$) and $K_m$ (30 μM) values in the presence of poly(C), the second phase may be associated with either the ATP hydrolysis step or a conformational change prior to ATP hydrolysis.

To determine whether the second phase was associated with ATP hydrolysis or a step prior to ATP hydrolysis, the binding of AMPPNP and ATP·S was measured. With the unhydrolyzable AMPPNP analog, only a time-dependent increase in protein fluorescence was observed (data not shown). The observed rate increased with [AMPPNP] linearly to provide an AMPPNP-binding rate of 0.017 ± 0.004 μM$^{-1}$ s$^{-1}$ (Table I). This rate is similar to that of AMPPNP binding in the absence of RNA. Thus, RNA does not stimulate the AMPPNP-binding rate. In addition, the protein conformational change that we observed upon ATP binding was not present upon AMPPNP binding. This might suggest that the protein conformational change is associated with ATP hydrolysis. However, the kinetics of ATP·S binding to the Rho-RNA complex were biphasic and similar to ATP. Mixing of Rho-RNA complex with ATP·S led to a transient increase in protein fluorescence followed by a decrease, similar to ATP binding (data not shown). The binding rate of ATP·S (2.3 μM$^{-1}$ s$^{-1}$) is similar to the ATP-binding rate, and the conformational change following nucleotide binding occurred with a $k_{cat}$ of 70 s$^{-1}$ and a $K_m$ of 30 μM (Table I). Thus, unlike AMPPNP, ATP·S seems to be a good analog of ATP. The results with ATP·S indicate that the second phase represents a protein conformational change that occurs before ATP hydrolysis. It is possible that the protein conformational change represents RNA contacting the secondary sites in the central channel of the Rho ring, because this step is necessary for the hydrolysis of ATP (9).

### Kinetics of ATP Binding to Rho_polydC

To explore the possibility that the protein conformational change following ATP binding is associated with the RNA contacting the secondary sites, we measured the kinetics of ATP binding to the Rho_polydC complex. It has been shown that polydC binds to the primary nucleic acid binding sites that reside in the N-terminal domain of the Rho protein, but not to the secondary RNA-binding sites in the central channel of the Rho ring (24). polydC does not stimulate the hydrolysis of ATP (9, 25). After mixing ATP with Rho_polydC in a stopped-flow instrument, a small increase in protein fluorescence was observed, but the ATP-induced conformational change characterized by the decrease in protein fluorescence was not observed (Fig. 2B). The lack of the second phase was not due to a defect in ATP binding to the Rho-DNcomplex, because (as shown below) mant-ATP binds to Rho-DNcomplex at a stimulated rate. Because the protein conformational change is observed only with the Rho-RNA complex, the experiments of ATP binding to RhoDNA support the idea that the protein conformational change is associated with the RNA binding to the secondary sites. 

### Mant-ATP as a Fluorescent ATP Analog

We have investigated here the use of mant-ATP (Fig. 3A) as a fluorescent analog of ATP to carry out sensitive nucleotide-binding measurements.
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mant-ATP. The emission spectrum of Rho protein overlaps with the excitation spectrum of the mant-nucleotide indicating the possibility of FRET.

Before using mant-ATP in nucleotide-binding kinetic experiments, we measured the $k_{cat}$ and $K_m$ of mant-ATP hydrolysis. No hydrolysis was observed in the absence of RNA similar to what is observed with ATP. In the presence of poly(C) RNA, the $k_{cat}$ of mant-ATP hydrolysis is $6.5 \text{ s}^{-1}$ at 18°C (Fig. 3C), which is 5- to 6-fold slower than the $k_{cat}$ of ATP hydrolysis under similar reaction conditions. The $K_m$ of mant-ATP (48 ± 6.6 μM) is similar to the ATP $K_m$ (30 μM). These results indicate that mant-ATP is a good ATP analog that can be used to study nucleotide binding.

Kinetics of Mant-ATP Binding to Rho—The kinetics of mant-ATP binding to Rho protein was measured by using the stopped-flow method via FRET. Rho protein was rapidly mixed with mant-ATP, and the time-dependent increase in fluorescence due to FRET was monitored by exciting Rho at 290 nm and monitoring mant-ATP emission at longer wavelengths (440 nm $< \lambda_{em} <$ 460 nm). The kinetics of mant-ATP binding was biphasic (Fig. 4A). The fast phase rate increased linearly with mant-ATP concentration (Fig. 4B), with a slope of 0.07 ± 0.007 μM$^{-1}$ s$^{-1}$ and a y-intercept of 0.9 ± 0.1 s$^{-1}$. The slope of the fast phase dependence is very close to the bimolecular rate of ATP binding (Table I).

Similar biphasic kinetics but at faster rates was observed for mant-ATP binding to the Rho(poly(C)) complex (Fig. 5A). The fast phase of the observed biphasic kinetics increased linearly (Fig. 5B) with a slope of 3.6 ± 0.1 μM$^{-1}$ s$^{-1}$ and a y-intercept of 6.2 ± 1.5 s$^{-1}$. These values are very similar to the fast phase of ATP binding in the presence of RNA (Table I). Thus, it is clear that the fast phase represents the initial binding of mant-ATP to Rho or the Rho-RNA complex. However, the origin of the slow phase is less clear.

The slow phase could represent a conformational change in Rho upon mant-ATP binding. Alternatively, we considered the possibility that the two phases in mant-ATP binding arise from the two isomers of mant-ATP; that is, the mant group linked to the 2'-hydroxyl $versus$ the 3'-hydroxyl group. It is known that the synthesis of mant-ATP provides an equilibrium mixture of 2'- and 3'-isomer of mant-ATP with a yield of 35 and 65%, respectively (26). Therefore, we investigated the interactions of Rho with two additional mant-nucleotide derivatives, where the fluorophore was attached specifically to the 3'-hydroxyl (3'-mant-2'deoxy) or the 2'-hydroxyl (2'-mant-3'deoxy) of the ribose ring. Both the mant-dATP analogs bind to Rho(poly(C)) in a single kinetic phase (Fig. 5, C and E). The observed rates increased linearly with [nucleotide], as shown in Fig. 5, D and F. The slopes indicated that the 3'-mant-2'dATP binds slightly faster than the 2'-mant-3'dATP (Table I). Thus, it seems that the two phases in mant-ATP binding kinetics are due to the slightly different binding properties of the 2'- and 3'-isomers. Mant-ATP binding was also measured to the RhoC$_{100}$ complex. The kinetics of nucleotide binding is fast when DNA is complexed to the Rho protein. The fast phase rate increased linearly with nucleotide concentration to provide a slope of 0.5 ± 0.1 μM$^{-1}$ s$^{-1}$ and a y-intercept of 5.5 ± 1.9 s$^{-1}$ (Table I). Thus, the binding of dC$_{100}$ to the Rho hexamer increases the nucleotide-binding rate about 7-fold.

Kinetics of Mant-ATP Dissociation from Rho—To determine the effect of RNA on the dissociation kinetics of nucleotide, we measured the off rate of mant-ATP in the absence and in the presence of RNA. Mant-ATP was pre-mixed with Rho and RNA for a short time (t$=10$ ms to 1.0 s) using the three-syringe set up of the stopped-flow instrument to minimize mant-ATP hydrolysis (Fig. 6A for the double-mixing setup). After the short
preincubation period, Rho RNA/mant-ATP complex was chased with excess ATP. Dissociation of mant-ATP from the Rho RNA complex was determined by monitoring the mant-ATP fluorescence decrease after excitation at 345 nm. The dissociation kinetics best fit to the sum of two exponentials. B and C, the increase in the fast phase ($k_1$) and the slow phase ($k_2$) rate, respectively, with [mant-ATP]. Slopes and intercepts are listed in Table I.

**DISCUSSION**

The stopped-flow kinetics in this study show that the nucleotide-binding rates are much faster when Rho is complexed to RNA as compared with the RNA-free Rho protein. Nucleotides such as ATP, ATP$\gamma$S, and mant-ATP bind to the Rho RNA complex with similar association rates of the order of $10^8 \text{ M}^{-1}\text{s}^{-1}$. The RNA-free Rho protein, on the other hand, binds ATP and mant-ATP with a slow association rate of the order of $10^4 \text{ M}^{-1}\text{s}^{-1}$. The same trend was observed in the rate of nucleotide release from Rho. When RNA is bound to Rho, mant-nucleotide release is fast, whereas the RNA-free Rho slowly dissociates the bound mant-ATP. The 50- to 70-fold slower rate of nucleo-

**Fig. 4.** Stopped-flow kinetics of mant-ATP binding to RNA-free Rho protein monitored by FRET. A, Rho hexamer (20 nm) in Tris buffer at 18°C was rapidly mixed with mant-ATP, and the increase in fluorescence ($\lambda_{ex} = 290 \text{ nm}, 440 \text{ nm} < \lambda_{em} < 460 \text{ nm}$) was monitored with time. Representative traces are shown for 2 nm (○), 6 nm (□), 10 nm (△), 20 nm (○), 30 μm (●), and 50 μM (○) mant-ATP binding. The data fit best to the sum of two exponentials. B and C, the increase in the fast phase ($k_1$) and the slow phase ($k_2$) rate, respectively, with [mant-ATP]. Slopes and intercepts are listed in Table I.

**Fig. 5.** Stopped-flow kinetics of mant-ATP and mant-dATP binding to Rho RNA complex. Rho hexamer (75 nm) and poly(C) RNA (100 nm) in Tris buffer were rapidly mixed with mant-ATP or mant-dATP nucleotides in a stopped-flow instrument at 18°C. A, C, and E, representative traces of the time-dependent increase in fluorescence at 10 μM mant-ATP, 2'-mant-3' dATP, and 3'-mant-2' dATP, respectively. A, Mant-ATP binding fit to the sum of two exponentials. B, the linear increase in the observed rate of the fast phase with [mant-ATP]. Inset, increase in the rate of the slower phase with [mant-ATP]. Slopes and intercepts are listed in Table I. The binding of mant-dATP nucleotides fit to a single exponential. E and F, the plots of the observed rate versus the respective nucleotide concentration. The slopes and intercepts are listed in Table I.
tide binding and the 40- to 120-fold slower rate of nucleotide release in the absence of RNA indicates that the structure of RNA-free Rho is different from the one complexed with the RNA cofactor (Fig. 7). It is known that the RNA wraps around and binds with a high affinity to the primary RNA-binding sites in the N-terminal domain of Rho (3, 27–29). Our results indicate that RNA binding to the primary sites causes a conformational change in the quaternary structure of Rho that exposes the nucleotide-binding sites at the subunit interface and renders them more accessible to the incoming and outgoing nucleotides (Fig. 7). This is in agreement with the result that the mant-ATP binding rate is stimulated by dC100, which binds only to the primary sites. The association rate of mant-ATP for the Rho/poly(dC) complex is, however, only 7-fold greater than the association rate of mant-ATP to free Rho, whereas the association rate of mant-ATP for the Rho/poly(C) complex is 50- to 70-fold greater. Hence, the complex of Rho with RNA must be structurally distinct from the complex of Rho with DNA. However, this was not evident from the crystal structure of the Rho hexamer with oligo(C) and oligo(dC) (10).

The studies of nucleotide binding in this paper and the previous studies of RNA binding (13) indicate that there is cooperativity between the nucleic acid and the nucleotide-binding sites. When RNA is bound to Rho, the ATP-binding kinetics are biphasic, and the experiments in this study provide evidence for a protein conformational change following ATP binding that results in RNA binding to the secondary-binding sites. The protein conformational change was observed with ATP but not the unhydrolyzable ATP analog, AMPPNP. Thus, conformational changes in the nucleic acid binding site are caused by specific interactions of the nucleotide with the Rho hexamer. Similarly, DNA does not stimulate the rate of nucleotide bind-
ing to the extent that RNA does, which indicates that DNA and RNA induces distinct changes in the nucleotide-binding sites. The protein conformational change as a result of ATP binding to the Rho-RNA complex occurs at about 47 s⁻¹, which is very close to the rate of RNA-stimulated ATP hydrolysis (35–40 s⁻¹). We propose that the Rho hexamer becomes competent in ATP hydrolysis and translocation upon undergoing this conformational change.

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