Escherichia coli transcription termination factor rho is a hexamer with three catalytic subunits that turnover ATP at a fast rate and three noncatalytic subunits that turnover ATP at a relatively slow rate. The mechanism of the ATPase reaction at the noncatalytic sites was determined and was compared with the ATPase mechanism at the catalytic sites. A sequential mechanism for ATP binding or hydrolysis that was proposed for the catalytic sites was not observed at the noncatalytic sites. Pre-steady-state pulse-chase experiments showed that three ATPs were tightly bound to the noncatalytic sites and these were simultaneously hydrolyzed at a rate of 1.8 s⁻¹ at 18 °C. The apparent bimolecular rate constant for ATP binding was determined as 5.4 × 10⁷ M⁻¹ s⁻¹ in the presence of poly(C) RNA. The ATP hydrolysis products dissociated from the noncatalytic sites at 0.02 s⁻¹. The hydrolysis of ATP at the noncatalytic sites was at least 130 times slower, and the overall ATPase turnover was 1500 times slower than that at the catalytic sites. These results from studies of the rho protein are likely to be general to hexameric helicases. We propose that the ATPase activity at the noncatalytic sites is too slow to drive translocation of the protein on the nucleic acid or to provide energy for nucleic acid unwinding.

The Escherichia coli rho protein is a transcription termination factor that is required for the release of certain nascent RNAs from the transcription complex (1, 2). Rho has various activities such as RNA binding (3), RNA-dependent ATP hydrolysis (4, 5), and an ATPase-dependent helicase activity that unwinds RNA-DNA duplexes (6). The rho protein functions as a hexamer of identical subunits (7) and shows structural and mechanistic similarities to hexameric DNA helicases such as E. coli DnA3 and the T7 gene 4 helicase (8, 9). The structure and mechanism of the rho protein appear to be similar to the αβδ F₁-ATPase protein. The tertiary structure of the RNA-binding domain residing at the N terminus of rho is homologous to the structure of the N-terminal domain of the F₁-ATPase, despite there being no amino acid sequence homology between them (10). The C-terminal domain of rho shows significant amino acid homology to the β-subunit of F₁-ATPase. The amino acid sequence homology between rho and the β subunit is more striking than the amino acid sequence homology between the α and β subunits of the F₁-ATPase protein (11). In addition to the structural similarity, the ATPase mechanisms of both rho and the T7 gene 4 helicase show striking similarity to the binding change mechanism of the F₁-ATPase protein (9, 12).

Unlike the F₁-ATPase protein, the rho protein is a homohexamer. However, several studies suggest that the hexamer is asymmetric with a C₃ᵥ symmetry (13). This asymmetry results in three high affinity and three low affinity ATP binding sites (14, 15) and two classes of nucleic acid binding sites on the rho hexamer (16, 17). Our previous studies showed that the ATPase turnover rate was different at the two classes of nucleotide binding sites on the rho hexamer (18). In the presence of poly(C) RNA, the ATPase turnover at the high affinity noncatalytic sites was 1500 times slower than at the low affinity catalytic sites. In this paper, the mechanism of the ATPase reaction at the noncatalytic sites was determined using pre-steady-state kinetic approaches. This study allowed us to make a comparison between the mechanisms of ATP hydrolysis at the noncatalytic and the catalytic sites of the rho hexamer.

**MATERIALS AND METHODS**

**Protein, Nucleotides, RNA Homopolymer, and Other Reagents—**Purified rho protein was provided by Dr. Katsumi Shigasada (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 (19) and purified according to Finger and Richardson (20) with slight modifications. The rho protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of 0.325 (mg/ml)⁻¹ cm⁻¹ (7). The ATP and RNA homopolymer, poly(C), was purchased from Amersham Pharmacia Biotech. Poly(C) RNA had a reported s₂₀,w 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 269 nm using an extinction coefficient of 6200 M⁻¹ cm⁻¹ for the cytosine base. These RNAs were 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 polyethyleneimine-cellulose TLC and corrected for in all experiments.

**The Three Syringe Rapid Chemical Quench-Flow Experiments—**The kinetic experiments were conducted using a rapid chemical quench-flow instrument (21) built by KinTek Corp. (State College, PA) at a constant temperature of 18 or 10 °C. In the three syringe experimental set-up, two delay times were used (KinTek RQF-3 software) as shown in Scheme I. The pulse-chase kinetics were measured at fixed t₁ and varying t₂ (0.015–7 s). A 14-μl mixture of rho protein (2.0 μM hexamer) and poly(C) RNA (2.68 μM) in 40 mM Tris-HCl (pH 7.8), 100 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol, and 10% (v/v) glycerol was rapidly mixed with a 14-μl mixture of ATP (100 μM) and [α-³²P]ATP (0.03 μCi/μl) for t₁. After reacting the above for t₁, 31 μl of nonradioactive ATP (5 mM, final concentration after mixing and 10 μM free MgCl₂) was added from a third syringe in the quench-flow instrument. The final unlabelled ATP concentration exceeded the labeled ATP concentration by at least 200-fold. A total of 59-μl reactions were chased for varying times (t₂) and quenched with 120 μl of 0.5 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 0.3 M potassium phosphate, pH 3.4. The resolved radioactive ATP and ADP were quantitated on a PhosphorImager instrument (Molecular Dynamics). Product formation was equal to the radioactive corresponding to ADP divided by the total radioactivity. To estimate ATP hydrolyzed in the reaction time (t₁) prior to the ATP...
chase, 0.5 M formic acid was added from the third syringe instead of the ATP chase. This control experiment was repeated three times, and the values were averaged and taken as a zero time point in the chase-time period ($\Delta t_2$). The efficiency of 0.5 M formic acid as a quench was determined by loading the rho protein/RNA solution into one sample loop, the reaction buffer into the other sample loop, the quench solution for 5 ms prior to mixing with the ATP solution. Only 0.03% of ATP was hydrolyzed to ADP, which was the same as the level observed in the absence of enzyme. This indicates that the quench solution inactivated the enzyme within 5 ms.

Data Analysis—The hydrolysis of radioactive ATP during the chase-time, $t_2$, was corrected as follows. A correction factor, $B$, was calculated,

$$B = \frac{[E]_0 \times D \times k_{\text{cat}} \times [\text{ATP}]_0 \times [\text{M}]}{[\text{ATP}]_0}$$

where $[E]_0$ (1.0 $\mu$M) is the concentration of rho hexamer before chase, $D$ (28 $\mu$L/59 $\mu$L) is the dilution factor because of the addition of chase, $[\text{ATP}]_0$ (50 $\mu$M) is the concentration of radioactive ATP before chase, $k_{\text{cat}}$ is the ATPase turnover number (30 and 8.8 s$^{-1}$ at 18 and 10 °C, respectively), and $[\text{M}]$ (5.0 mM) is the concentration of chase ATP. The value of $B \times t_2$ was subtracted from each data point in the pulse-chase experiments. The corrected amounts of ADP formed during the chase-time $t_2$ were plotted and were fit to the following single exponential equation with a y intercept using the Sigma Plot software version 5.0 (SPSS).

$$[\text{ADP}]_2 = [\text{ADP}]_1 + A (1 - e^{-t_2/t_1})$$

Where $t_1$ and $t_2$ are the two delay times (see Scheme 1), $[\text{ADP}]_1$ is the amount of ADP formed during the reaction time ($t_1$), $[\text{ADP}]_2$ is the total amount of ADP formed, $k_1$ is the rate of ATP hydrolysis during chase-time $t_2$, and $A$ represents the amount of ADP formed during chase-time $t_2$.

RESULTS AND DISCUSSION

The two classes of ATP binding sites on the rho hexamer termed catalytic and noncatalytic sites hydrolyze ATP at different rates. At the catalytic sites, close to 30 ATPs are hydrolyzed/mole of rho hexamer/second, and our previous studies have shown that the ATPase turnover at the catalytic sites is about 1500 times slower (18). The fast ATPase turnover at the catalytic sites precludes measurement of the slow ATPase reaction at the noncatalytic sites. To dissect the ATPase kinetics at the noncatalytic sites, we took advantage of the tight binding of ATP at the noncatalytic sites and used pulse-chase experiments to determine the kinetics of ATP binding and hydrolysis at those sites.

Kinetics of ATP Hydrolysis at the Noncatalytic Sites of Rho—

Scheme I shows the design of the pulse-chase experiments. A complex of rho protein and poly(C) RNA (2.88 $\mu$M) was mixed with $[\alpha^{32}\text{P}]}$ATP (100 $\mu$M) for a fixed pulse period $t_1$. The reaction was then chased with 5 mM (final concentration after mixing) of nonradioactive ATP, and the chase-time $t_2$ was varied from 0.015 to 7.0 s. The experiment was carried out in a rapid quench-flow instrument at 18 °C as shown in Scheme 1. The filled circles (●) in both panels show the amount of ATP hydrolyzed in the pulse period $t_1$. The data were corrected for radioactive ATP hydrolysis in the chase-time (“Materials and Methods”). The left y axis shows the corrected amount of ATP hydrolysis during the chase-time, which was obtained by subtracting the amount of ATP hydrolyzed during pulse-time $t_1$ and divided by [rho]. The right y axis shows the uncorrected ATP hydrolysis. Insets in both panels show the time-course up to 500 ms. A, the pulse-chase kinetics of ATP hydrolysis with pulse-time $t_1$ fixed at 100 ms. Computer fit of the kinetics to Equation 2 (“Materials and Methods”) represented by a solid line showed that 3.8 ± 0.5 ATPs were hydrolyzed to product during the 100 ms pulse-time. One ATP (0.96 ± 0.23 ATPs/hexamer) was hydrolyzed at a fast rate, and three ATPs (2.80 ± 0.31 ATPs/hexamer) were hydrolyzed at a rate of 1.9 ± 0.6 s$^{-1}$. B, the pulse-chase kinetics of ATP hydrolysis with pulse-time $t_1$ fixed at 30 ms. Under these conditions, a total of 1.8 ATPs were hydrolyzed to product/rho hexamer. Less than one ATP (0.51 ± 0.13 ATPs/hexamer) was hydrolyzed at a fast rate, and 1.3 ± 0.1 ATPs/hexamer were hydrolyzed at a rate of 2.31 ± 0.81 s$^{-1}$.

Fig. 1A shows the results of such an experiment where pulse-time $t_1$ was fixed to 0.1 s, and the chase-time $t_2$ was varied from 0.015–7.0 s. There are two exponential phases in the pulse-chase time course. Because of the lack of time points shorter than 15 ms, the fast exponential phase could not be defined. Hence, the data were fit to a single exponential with a y intercept (Equation 2) instead of the sum of two exponentials. This fit provided the rate constant of the slow phase and the rate of ATP hydrolysis to ADP and $P_1$ dictated by the respective rates of ATP dissociation and ATP hydrolysis on the protein. The radioactive ATP that dissociates from rho cannot rebind because it will be diluted in the pool of excess nonradioactive ATP. From the kinetics of radioactive ADP formation in the chase-time $t_2$, one can estimate both the number of ATP molecules that remain bound and chased to product as well as their rate of hydrolysis.

Fig. 1A shows the results of such an experiment where pulse-time $t_1$ was fixed to 0.1 s, and the chase-time $t_2$ was varied from 0.015–7.0 s. There are two exponential phases in the pulse-chase time course. Because of the lack of time points shorter than 15 ms, the fast exponential phase could not be defined. Hence, the data were fit to a single exponential with a y intercept (Equation 2) instead of the sum of two exponentials. This fit provided the rate constant of the slow phase and the rate of ATP hydrolysis to ADP and $P_1$.
amplitudes of both the slow and the fast phase. A total of four ATPs were chased to ADP during the chase-period, indicating that at least four ATPs were bound in the pulse-time of 0.1 s. Of the four ATPs, one was hydrolyzed at a very fast rate estimated to be $>150 \text{ s}^{-1}$ in the chase-time $t_2$ (assuming that the first time point of 15 ms represented 3–4 half-lives), and the rest were hydrolyzed at a slow rate equal to 1.9 s$^{-1}$.

The ATP that was hydrolyzed at a rate greater than the steady-state ATPase turnover was most likely bound to one of the catalytic sites. The remaining three ATPs must be bound to the noncatalytic sites, because these were hydrolyzed at a rate 15 times slower than the steady-state ATPase rate of 30 s$^{-1}$. Similar kinetics were observed when the pulse-time $t_2$ was changed to 30 ms (Fig. 1B). Only the total number of ATPs chased to product was different. After a 30-ms pulse, a total of 1.8 radioactive ATPs/rho hexamer were chased to products. Of the 1.8 ATPs, less than 1 ATP (0.51 ATP) was hydrolyzed to ADP at a fast rate and about 1.3 ATPs were hydrolyzed at a slow rate. These results indicate that only a limited number of ATPs were bound to the noncatalytic sites during the pulse-time of 30 ms. This result prompted us to carry out a systematic study in which the pulse-chase kinetics were measured at various fixed pulse-times to measure the apparent rate of ATP binding to the noncatalytic sites.

**Kinetics of ATP Binding to the Noncatalytic Sites of Rho**—Examination of the pulse-chase kinetics at varying pulse-times provided an estimate of the apparent bimolecular rate of ATP binding to the noncatalytic sites. The chase kinetics were measured at various pulse-times $t_2$ that ranged from 10 to 150 ms. Each chase-time course was analyzed to obtain the number of ATPs bound to the noncatalytic and the catalytic sites and the rate of ATP hydrolysis at the noncatalytic sites. These parameters were obtained from computer fit of the data to a single exponential with a $y$ intercept (Equation 2 under “Materials and Methods”). As the pulse-time $t_2$ was increased from 10 to 150 ms, the total number of ATPs chased to product increased to a maximum of three to four. As shown in Fig. 2A, during a 10–150-ms pulse-time, less than one ATP (0.60 ± 0.08) was hydrolyzed at a very fast rate (within the fastest time point of 15 ms). The number of ATPs that were more slowly hydrolyzed to product increased from one to a maximum of three ATPs (2.8 ± 0.2) as the pulse-time $t_2$ was increased (Fig. 2B). This increase in ATP site occupation at the noncatalytic sites was exponential and fit to a rate constant of $27 \text{ s}^{-1}$. The rate of slow ATP hydrolysis remained constant around an average value of $1.8 \text{ s}^{-1}$ (Fig. 2C). We assume that the fast hydrolysis of ATP at one site occurs at the catalytic site, and the slow hydrolysis of ATP at the noncatalytic sites as $2.80 \pm 0.2$ C, the $t_1$ dependence of the number of ATPs hydrolyzed to product at the catalytic sites ($y$ intercept). An average of $0.60 \pm 0.08$ ATP were hydrolyzed to product at the catalytic sites (dashed line). B, shows the $t_1$ dependence of the ATP bound to the noncatalytic sites. The site occupancy at the noncatalytic sites increased in an exponential manner with a rate constant of $26.8 \pm 5.6 \text{ s}^{-1}$ (solid line). The amplitude provided the maximum number of ATPs hydrolyzed to product at the noncatalytic sites as $2.80 \pm 0.2$. C, the $t_1$ dependence of the rate of ATP hydrolysis at the noncatalytic sites. The ATP hydrolysis rate at the noncatalytic sites remained constant around $1.84 \pm 0.19 \text{ s}^{-1}$ (dashed line).

**The Rate of ATP Hydrolysis at the Catalytic Site of Rho**—In the pulse-chase experiments described above, we found that the ATP bound at the catalytic site was completely hydrolyzed to ADP in the shortest chase-time of 15 ms. Thus, we could only estimate its rate of hydrolysis at $>150 \text{ s}^{-1}$. To obtain a more accurate value for the rate of ATP hydrolysis at the catalytic site, we performed the pulse-chase experiment both at a lower temperature of 10 °C and at a higher concentration of rho. Fig. 3 shows the results of the pulse-chase experiment in which the pulse-time was fixed at 0.3 s and the chase-time was varied. The same kinetic pattern for hydrolysis of ATP was observed at the lower temperature. One ATP was hydrolyzed at a fast rate and two to three (2.2 ± 0.3 ATPs) were hydrolyzed at a slow rate. The ATP hydrolysis at the catalytic site was still too fast to measure. Most of the ATP at the catalytic site was hydrolyzed in the fastest time of 15 ms providing an estimate of $>150 \text{ s}^{-1}$ for the hydrolysis rate at 10 °C. The ATP hydrolysis rate at
the noncatalytic sites decreased to a value of 1.12 s$^{-1}$, about 1.6 times slower than the rate of hydrolysis at 18 °C. Assuming the same temperature dependence for the catalytic sites, we estimate that ATP hydrolysis at the catalytic sites occurs at a rate >240 s$^{-1}$ at 18 °C.

**A Minimal Mechanism For ATP Binding and Hydrolysis at the Catalytic and Noncatalytic Sites of Rho**—The results presented in this paper provide a kinetic pathway for the ATPase reaction at the noncatalytic sites of the rho hexamer. Combined with the recently published model of ATPase reaction at the catalytic site (12), it allows us to make a comparison between the ATPase reaction pathway at the catalytic versus the noncatalytic sites (Fig. 4). Although the two pathways are shown as independent ones, note that they occur simultaneously at the two types of sites on the rho hexamer. Fig. 4A summarizes the ATPase reaction at the noncatalytic sites. Three ATPs bind to the noncatalytic sites of rho with an apparent bimolecular rate constant of $5.4 \times 10^5$ M$^{-1}$ s$^{-1}$. These ATPs are hydrolyzed at a rate of about 1.8 s$^{-1}$, and the products, ADP and/or P$i$, dissociate from the active site at a rate of 0.02 s$^{-1}$. The dissociation rate was obtained from previous studies (18). In contrast, hydrolysis of ATP has been proposed to be sequential at the catalytic sites as shown in Fig. 4B (12). The rho-ATP complex at the catalytic site is formed with an apparent bimolecular rate of $3 \times 10^6$ M$^{-1}$ s$^{-1}$, estimated from the ratio $k_{cat}/K_{M}$. Only one ATP is hydrolyzed at a time at the catalytic site. This rate is estimated at >240 s$^{-1}$ from this study as well as a published study on the ATPase kinetics (12). In that report (12), a pre-steady-state burst of ATP hydrolysis was observed, implying that a step following ATP hydrolysis is a rate-limiting step. After the rate-limiting step, the next catalytic subunit presumably goes through the same cycle of ATP binding and hydrolysis. When we compare the ATPase kinetics at the two types of sites, it is clear that sequential hydrolysis of ATP does not occur at the noncatalytic sites. The results in this paper show that all three ATPs are hydrolyzed simultaneously at the noncatalytic sites at a slow rate relative to the catalytic site (Fig. 2, B and C). Hence, there does not appear to be any cooperativity in ATP binding and hydrolysis at the noncatalytic sites. The rate of ATP hydrolysis is at least 130 times slower (>240 s$^{-1}$/1.8 s$^{-1}$), and product release is 1500 times slower at the noncatalytic sites compared with the corresponding steps at the catalytic site, assuming that $k_{cat}$ is largely determined by the product release step at the catalytic sites. This indicates that the ATP and/or the ADP and P$i$ species are bound to the noncatalytic sites, whereas multiple ATPase turnovers occur at the catalytic sites.

**Role of the Noncatalytic Sites**—Noncatalytic sites have been identified thus far in three hexameric proteins including rho protein, the T7 gene 4 helicase (8), and the F$_1$-ATPase (22, 23). In the $c_6b_2$ structure of F$_1$-ATPase, the noncatalytic sites reside primarily on the $\alpha$ subunits. The ATP binding site of the $\alpha$ subunits lacks critical amino acids that are necessary for catalysis of ATP hydrolysis. Thus, hydrolysis of ATP at the noncatalytic sites of F$_1$-ATPase is not easily detectable. Because rho is a homohexamer, the catalytic and noncatalytic active sites must be induced either during hexamer formation or upon ATP and/or RNA binding. If the catalytic and noncatalytic active sites of rho contain the same amino acids, then the observed difference in the ATPase rates at the two sites suggests that the conformation of some of the critical amino acid residues that are involved in ATP hydrolysis are different. This hypothesis will be tested when the high resolution structure of rho hexamer in the presence of ATP is available.

The noncatalytic sites were first identified in the F$_1$-ATPase protein. However, the exact role of the noncatalytic sites in F$_1$-ATPase is still unclear. Nevertheless several studies indicate that the noncatalytic sites are essential for optimal ATPase activity. It has been suggested that the fully functional noncatalytic sites in F$_1$-ATPase are necessary for maintaining cooperative catalysis at the catalytic sites. When wild-type $\alpha$ subunits in F$_1$-ATPase hexamer were replaced with mutant $\alpha$ subunits defective in ATP binding, the ATP hydrolysis at the catalytic sites was inhibited in a time-dependent manner (24). This time-dependent inhibition was shown to be because of tight binding of inhibitory Mg-ADP at the catalytic sites. These results suggest that the fully functional noncatalytic sites in F$_1$-ATPase are required for continuous ATPase turnover at the catalytic sites.

Two experiments withrho suggest that the noncatalytic sites in rho are essential. When two to three subunits of the wild-type rho hexamer were exchanged for an inactive mutant protein, a loss in the ATPase activity of rho was observed (25). Some of these substituted subunits could be the noncatalytic subunits, but it can be argued also that this inhibition was because of the loss in the function of the catalytic sites. Inactivation of the rho ATPase was also observed when one rho subunit was covalently modified with 8-azido-ATP (26). Assuming that the catalytic and noncatalytic sites have an equal chance of being covalently modified, the results argue that modification of the noncatalytic sites inhibits ATP hydrolysis at the catalytic sites.

Is the hydrolysis of ATP at the noncatalytic sites coupled to movement on the RNA? To address this question, we have calculated the coupling constants (nt moved/ATP hydrolyzed) for the catalytic and noncatalytic sites. Based on these numbers, we conclude that the conformational changes resulting from the hydrolysis of ATP at the noncatalytic sites cannot be directly coupled to movement. Walstrom et al. (27, 28) have reported that rho translocates along the RNA at a rate of ~20 nt/s and consumes about 1–2 ATP when translocating about 1 nucleotide of RNA. This provides a coupling constant of about 0.5–1 nt/ATP hydrolyzed for the catalytic sites. Similarly, Brennan et al. (29) have reported a coupling constant close to 0.06 nt/ATP hydrolyzed. The ATP turnover at the noncatalytic sites of rho occurs at 0.02 s$^{-1}$ (18). To calculate the coupling
constant for the noncatalytic sites, we have corrected the above translocation rate (decreased 4-fold) because that number was measured at 37°C and the turnover number of 0.02 s\(^{-1}\) at the noncatalytic sites was measured at 18°C. According to this rough calculation, if the hydrolysis at the noncatalytic sites were directly coupled to movement of rho along the RNA, then the rho protein will move close to 250 nt/ATP hydrolyzed. We believe that this is unlikely because rho binds only about 60–70 nt of RNA (30). We therefore propose that the energy from ATP hydrolysis at the noncatalytic sites alone is not utilized directly for translocation or for nucleic acid unwinding. At this time, the role of ATP binding or its hydrolysis at the noncatalytic sites is unclear. The ATP at the noncatalytic sites may play a regulatory role analogous to that proposed for the noncatalytic sites of F\(_{1}\)-ATPase; that is, these sites mediate cooperative catalysis of ATP at the catalytic sites and/or ensure continuous ATP hydrolysis at the catalytic sites. Alternatively, the noncatalytic subunits may participate in interactions with the nucleic acid during active ATP hydrolysis at the catalytic site and thus facilitate processive translocation of the protein on the nucleic acid.

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