The active form of transcription termination factrhorho from Escherichia coli is a homohexamer, but several studies suggest that the six subunits of the hexamer are not functionally identical. Rho has three tight and three weak ATP binding sites. Based on our findings, we propose that the tight nucleotide binding sites are noncatalytic and the weak sites are catalytic. In the presence of RNA, the rho-catalyzed ATPase rate is fast, close to 30 s^{-1}. However, under these conditions the three tightly bound nucleotides dissociate from the rho hexamer at a slow rate of 0.02 s^{-1}, indicating that the three tight nucleotide binding sites of rho do not participate in the fast ATPase turnover. These slowly exchanging nucleotide binding sites of rho are capable of hydrolyzing ATP, but the resulting products (ADP and P_i) bind tightly and dissociate from rho about 1500 times slower than the fast ATPase turnover. Both RNA and excess ATP in solution are necessary for stabilizing nucleotide binding at these sites. In the absence of RNA, or when solution ATP is hydrolyzed to ADP, a faster dissociation of nucleotides was observed. Based on these results, we propose that the rho hexamer is similar to the F_1-ATPase and T7 DNA helicase-containing noncatalytic sites that do not participate in the fast ATPase turnover. We propose that the three tight sites on rho are the noncatalytic sites and the three weak sites are the catalytic sites.

The transcription termination factor rho is an essential protein in Escherichia coli that is required for the release of certain nascent RNAs from the transcription complex (1–4). The rho protein contains an ATPase-dependent helicase activity that can separate the strands of an RNA-DNA duplex. Transcription termination by rho starts after the rho protein binds to the nascent RNA transcript at a cytosine-rich entry site (5). The RNA binding event activates the ATPase activity of rho, which is believed to fuel a directional 5' → 3' movement of the protein along the nascent RNA chain. Both the ATPase and the helicase activities are necessary for the rho-dependent termination event (6). Thus, it is believed that translocation and RNA-DNA duplex unwinding activities cause disruption of the elongating complex, which results in transcription termination (7–9).

Several studies of the rho protein have shown that it assembles into a planar hexagonal structure that contains a central hole (10, 11). The self-association of rho subunits into a hexamer is reversible in solution, and stable hexamer is formed in the presence of ATP and/or RNA (12, 13). Each subunit of rho hexamer contains two functionally distinct domains, an ATP- and an RNA-binding domain (1). The ATP-binding domain of rho shows greater than 50% amino acid sequence homology to the β subunit of F_1-ATPase (14). The tertiary structure of the RNA-binding domain of rho is also very similar to the N-terminal domain of F_1-ATPase, despite little amino acid sequence homology between these domains of the two proteins (15). A recent report has also shown similarities among the ATPase mechanism of rho, the F_1-ATPase, and T7 DNA helicase (23, 24). These observations support the proposal that the structure of the rho hexamer is likely to be similar to that of the F_1-ATPase (14–16).

Several studies in the literature indicate that the rho hexamer is not symmetrical. Geiselmann et al. (17) suggested a D_3 symmetry for the rho hexamer, and recently Horiguchi et al. (18), using extensive chemical cross-linking experiments, have suggested a C_3 or a C_{3v} symmetry for the rho quaternary geometry. Equilibrium nucleotide binding experiments have shown that the rho hexamer binds only three ATP molecules with a high affinity (19). Others have shown that in addition to these high affinity sites, there are three low affinity ATP binding sites on rho (20, 21). Thus, rho protein falls into the class of hexameric helicases, such as the E. coli DnaB (22) and T7 DNA helicase (23) that were shown to contain two classes of nucleotide binding sites. The three tight nucleotide binding sites on T7 DNA helicase hexamer were shown to be noncatalytic, with properties similar to such sites of the F_1-ATPase. Because rho protein is structurally similar to the F_1-ATPase protein, we have investigated the possibility of noncatalytic sites on the rho hexamer.

Our results show that the two classes of nucleotide binding sites on rho catalyze ATPase turnover at different rates. The ATPase turnover rate at one class of nucleotide binding sites was about 1500 times slower than the turnover rate at the other sites. These studies allow a better understanding of the nature of the two classes of nucleotide binding sites on the rho hexamer. We propose that the three tight nucleotide binding sites on the rho hexamer are noncatalytic, serving a role similar to such sites of the F_1-ATPase and T7 DNA helicase, and that the weak sites are the catalytic sites.

MATERIALS AND METHODS

Rho Protein—Rho protein was overexpressed in E. coli strain HB101 carrying the rho overexpression plasmid pKS26 (25) and purified according to Finger and Richardson (26) with slight modifications. Briefly, protein extracts from heat-induced cells were subjected to fractionation with Polyvin P, ammonium sulfate precipitation, and successive chromatography on SP-Sepharose FF and heparin-Sepharose (Amersham Pharmacia Biotech). The final preparation was stored at −20 °C in rora storage buffer containing 50% glycerol, 20 mM Tris-HCl (pH 7.9), 100
mm KCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Rho protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of 0.325 (mg/ml)⁻¹ cm⁻¹ (11).

Nucleotides, RNA Homopolymers, and Other Reagents—ATP and RNA homopolymers (poly(C) and poly(U)) were purchased from Amersham Pharmacia Biotech, and poly(C) RNA had a reported s_{20, w} value of 7.1 in 0.015 M NaCl, 0.0015 M sodium citrate buffer, pH 7.0, with an average length of 420 bases. Poly(U) had an s_{20, w} value of 6.0, with an average length of 290 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, and poly(U) RNA concentration at 260 nm, using the dissociation rate of 0.15 M for uracil base.

These RNAs were dissolved in TE buffer (40 mM Tris-HCl, pH 7.5, 0.5 mM EDTA) and used without further purification. [γ-³²P]ATP and [α-³²P]ATP were purchased from Amersham Pharmacia Biotech, and their purity was assessed by polyethyleneimine (PEI)-cellulose TLC and corrected for in all experiments. The ATP-regenerating reagents, phosphocreatine and creatine kinase, were purchased from Roche Molecular Biochemicals.

Equilibrium Binding of ATP to Rho Protein—Equilibrium binding of ATP to rho was measured using the nitrocellulose membrane binding assay. The nitrocellulose membrane circles (25 mm) were treated with 0.5 nM NaOH, rinsed extensively with water, and equilibrated in the membrane wash buffer (40 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM KC1, 0.1 mM dithiothreitol) before use. The reaction (15 µl) contained 0.5–500 µM radiolabeled ATP (α-³²P)ATP in binding buffer (40 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 50 mM KCl, 0.1 mM dithiothreitol, and 10% (v/v) glycerol). Rho protein was added to a final concentration of 1.0 µM (hexamer) and the reactions were incubated for 30 s at 18 °C. 12 µl of samples were then filtered through the nitrocellulose membrane assembly. The membranes were washed with 0.5 ml of wash buffer before and after filtration. 1-µl aliquots of each sample were spotted on a separate nitrocellulose membrane to quantitate total ATP. The radioactivity on the membrane was quantitated on a PhosphorImager (Molecular Dynamics). The molar amount of ATP bound to each rho hexamer was determined and plotted versus ATP concentration. The resulting titration data were fit using a hyperbolic equation to obtain the apparent K_{d} of rho-ATP complex.

ATPase Activity—The ATPase activity of rho protein was measured at 18 °C. The rho protein was incubated in the ATPase reaction buffer (40 mM Tris-HCl, pH 7.8, 100 mM KCl, 10 mM MgCl₂, 0.1 mM dithiothreitol and 10% (v/v) glycerol) in the presence of poly(C) or poly(U) RNA. Trace amounts of a >90% pure [γ-³²P]ATP (3000 Ci/mmol), together with nonradioactive ATP (1.0–3.0 mM), were added to start the reaction (30–50-µl reaction volumes). At various times, aliquots (5 µl) were withdrawn and quenched with 10 µl of 1 M HCl and 20 µl of chloroform. Quenched reactions were neutralized by adding 1 M NaOH, 0.25 M Tris base (about 9 µl), and 1 µl of the quenched reactions was spotted on a PEI-cellulose thin layer chromatography plate (Whatman). The radioactive ATP and P_i were separated using 0.3 M potassium phosphate, pH 3.4, as the chromatography running buffer. The ATP and P_i spots were quantitated using the PhosphorImager. The initial rates of P_i formation were determined from the decrease in radioactivity.

RESULTS

Interaction of Rho with ATP—To investigate the interaction of rho protein with ATP and to determine the number of ATPs bound to rho at equilibrium, we have used nitrocellulose filter binding assay. A constant amount of rho protein was titrated with increasing amounts of [α-³²P]ATP, and the rho-bound ATP was separated from free ATP by filtration through nitrocellulose membranes. As shown in Fig. 1, three ATP molecules were bound to rho at high ATP concentrations. The apparent K_{d} of rho-ATP complex was determined to be 17 µM. These results are consistent with studies that show that although rho has six identical ATP binding sites, rho binds only three ATPs at equilibrium (19–21). The ATPs bound to the weak sites are not easily detectable by this membrane filtration method, possibly because these ATPs dissociate during the filtration and washing steps.

We then explored the possibility of noncatalytic sites on rho. We designed experiments to determine whether the ATPs bound at the tight sites were catalytically competent. The experiment consisted of measuring the dissociation rate of nucleotides bound to the tight sites (k_{cat}) and comparing this value to the ATPase turnover rate, k_{cat}. If the tight sites are catalytically competent, then the k_{cat} should be equal to or faster than the steady state k_{cat}. If the k_{cat} from these sites is slower than the k_{cat}, then the ATPs bound to the tight sites cannot be catalytically competent and are likely to be noncatalytic.

The ATPase Turnover Rates in the Presence of Poly(C) and Poly(U) RNAs—The rho-catalyzed ATPase turnover rate was measured at 18 °C in the presence of poly(C) or poly(U) RNA. The concentration of RNA and ATP were kept at saturating concentrations to obtain the dissociation rate of rho-bound nucleotides. The chemical form of the nucleotides bound to rho was identified by extracting the nitrocellulose membrane filters with 0.5 M perchloric acid, neutralizing with 0.25 M Tris, 1 mM NaOH, and analyzing the extracted nucleotides by PEI-cellulose TLC.

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1 The abbreviation used is: PEI, polyethyleneimine.
ATPase $k_{\text{cat}}$ was only 7.2 mol of ATP hydrolyzed per s per rho hexamer. This rate appears to be lower because of the weaker affinity of rho for the poly(U) RNA. Accordingly, the ATPase $k_{\text{cat}}$ increased to 11.4 mol of ATP hydrolyzed per s per rho hexamer when the poly(U) RNA was increased to 0.33 $\mu$g/µl.

The Kinetics of Nucleotide Dissociation from the Rho Hexamer—The following experiment was designed to determine the dissociation rate of the three nucleotides bound to the rho protein at equilibrium. As shown in Scheme I, the experiment consisted of preincubating a complex of rho and RNA with radioactive ATP and then chasing the complex with an excess of nonradioactive ATP. The radioactive nucleotide bound to rho after various chase times were quantitated by the nitrocellulose filter binding assay. The results of such an experiment with poly(C) RNA and [$\alpha$-$^{32}$P]ATP are shown in Fig. 2A. Before chase was added, there were about 3 nucleotides bound per rho hexamer (Fig. 2A). After chase was added, all of the [$\alpha$-$^{32}$P]-labeled nucleotides dissociated from the rho protein with a single exponential rate of 0.02 ± 0.002 s$^{-1}$ (Fig. 2A). A similar nucleotide binding/exchange experiment, in the presence of poly(U) RNA at 0.2 $\mu$g/µl, provided a 10-fold faster nucleotide dissociation rate constant of 0.15 ± 0.02 s$^{-1}$ (Fig. 2B). When the poly(U) concentration was increased about 16-fold, the dissociation rate decreased to the same level as observed with poly(C) RNA (0.033 ± 0.005 s$^{-1}$ at 3.3 $\mu$g/µl of poly(U) RNA).

When the experiment was carried out in the absence of RNA (Fig. 2C), 3–4 nucleotides were bound to rho at equilibrium, in the absence of the chase. After chase was added, the [$\alpha$-$^{32}$P]-labeled nucleotides dissociated from the rho hexamer at a rate that was too fast to measure manually.

Table II compares the $k_{\text{off}}$ values of rho-bound nucleotides and the rho-catalyzed ATPase turnover rates. In the presence of poly(C) RNA, the $k_{\text{off}}$ of the three tight nucleotides was about 1500 times slower than the ATPase turnover rate. In the presence of poly(U) RNA, the $k_{\text{off}}$ of the nucleotides was about 50–340 times slower than the ATPase turnover rate. Thus the $k_{\text{off}}$ of the tightly bound nucleotides was consistently slower than the RNA-stimulated ATPase turnover rate. These results indicate that the nucleotides bound at the three tight sites on the rho hexamer do not participate in the fast ATPase turnover.

Identification of the Slowly Exchanging Nucleotides Bound at the Tight Sites of Rho—The above experiments indicated that the ATPs bound at the tight sites exchanged very slowly with solution ATP. The next question was whether the tightly bound nucleotides were ATP or ADP. In other words, does ATP get hydrolyzed at these tight sites? Two experiments were designed to determine the identity (ATP versus ADP) of the slowly exchanging nucleotides. These experiments were carried out in the presence of poly(C) RNA. Essentially the same experiment described above and shown in Fig. 2A was performed with [$\alpha$-$^{32}$P]ATP. After various chase times, the nitrocellulose membrane that contained the rho-bound radioactive nucleotide was extracted, and the radioactivity was analyzed by nitrocellulose TLC. As shown in Fig. 3, this experiment showed that most of the nucleotides bound to rho both before and after addition of the chase were ADP. The total amount of rho-bound ADP decreased with increasing chase time, as expected. A control experiment (Fig. 3, lane $\alpha$) showed that about one-third of the radioactive ATP was hydrolyzed with rho and RNA during the <10-s preincubation period. Therefore, the ATP substrate was not exhausted during the preincubation period.

![Scheme I](image)

**TABLE II**

| RNA | ATPase rate$^{a}$ | Off-rate, $k_{\text{off}}$$^{b}$ | Ratio ($k_{\text{cat}}$/$k_{\text{off}}$) |
|-----|------------------|------------------------------|---------------------------------|
| mol ADP/mol rho | nucleotide dissociation/s/rho | hexamer | hexamer | |
| Poly(C) | 30.2 ± 2.5 | 0.02 ± 0.002 | 1,510 |
| Poly(U) | 11.4 ± 1.2 | 0.033 ± 0.005 | 345 |
| No RNA | 7.2 ± 1.8 | 0.15 ± 0.02 | 48 |

$^{a}$ The steady-state ATPase turnover rates are taken from Table 1. $^{b}$ Off-rates of rho-bound nucleotides were obtained from the dissociation kinetic experiments shown in Fig. 2.

and the rho-bound radioactive ADP did not result from rebinding of radioactive ADP from solution. Similarly, we also determined that the acid extraction procedure resulted in less than
7% hydrolysis of ATP to ADP (Fig. 3, lane 11). The above results indicate that the tight sites are capable of hydrolyzing ATP, but the hydrolysis product dissociates from the tight sites at a slow rate.

To confirm these results, a second nucleotide binding/exchange experiment was carried out with \( \gamma^{32}\text{P}\)ATP instead of \( \alpha^{32}\text{P}\)ATP. If the ATPs are hydrolyzed at the tight nucleotide binding sites of rho and if the radioactive Pi does not remain tightly bound, then we should observe no detectable radioactivity on the nitrocellulose membranes in a reaction in which ATP was allowed to completely hydrolyze. Most of the nucleotide is ADP. Lane 10 shows a control in which radioactive ATP without rho was spotted on the membrane, which was acid-extracted. This procedure resulted in the isolation of 89% ATP. Because the commercial radioactive ATP contained about 4–6% ADP, very little ATP was hydrolyzed by the acid extraction procedure.

Cooperativity Between the Catalytic and the Noncatalytic Sites of Rho—Results thus far suggested that the ATPase turnover at the noncatalytic sites was about 1500 times slower than the ATPase turnover at the catalytic sites. To satisfy the first condition, the rate of nucleotide dissociation from the catalytic sites should be very slow. The hydrolysis products (ADP and Pi) remain tightly bound at these sites and dissociate at a slow rate, as shown in Fig. 5 (inset). Under these conditions, there were less than three radioactive nucleotides bound to rho at the tight sites, and these nucleotides dissociated at a rate that was too fast to manually measure. To satisfy the second condition, the same nucleotide binding/exchange experiment was carried out in the presence of an ATP regeneration system. Fig. 5, inset, shows that in the presence of the ATP regeneration system, the reaction mixture contained mostly ATP at the time chase was added. The three radioactive nucleotides that were bound prior to addition of the chase dissociated at a slow rate of 0.015 s\(^{-1}\) (Fig. 5). These results indicate that the three tight sites of rho retain their nucleotides at the active site as long as there is excess ATP in solution. Rho undergoes active ATP binding/hydrolysis events at the catalytic sites when ATP is present in solution. This active ATP binding and hydrolysis event at the catalytic sites appears to be necessary for retaining the ADP and Pi at the tight sites. These results suggest cooperativity among the two types of nucleotide binding sites of rho.
The catalytic sites of rho. The rate of ATPase turnover at the noncatalytic sites of the F1-ATPase protein bind ATP, and the noncatalytic sites also depend on the identity and the concentration of the RNA cofactor. The nucleotide dissociation rate constant was smallest from the rho-poly(C) complex, intermediate from the rho-poly(U) RNA complex, and fastest from rho uncomplexed with the RNA. The exact reason for this behavior is not known. If the nucleotides are bound at the interface of the rho subunits, as found in the F1-ATPase protein, then one might be able to provide a rationale for the above behavior, in terms of rho hexamer stability. A slower rate of nucleotide dissociation may occur from a more stable rho hexamer. It is known that poly(C) RNA binding to rho results in a stable rho hexamer (12, 27). In a stable hexamer, breathing or disruption of the subunit interfaces may be slow or occur less frequently, and thus nucleotide dissociation will be slower. This is consistent with the observation that nucleotides dissociate more slowly from poly(C) RNA-bound hexamer.

If the three tight sites on rho are noncatalytic, then the weak sites that are not detectable by filter binding or ultracentrifugation methods must be the catalytic sites. By performing ATP binding experiments at higher concentrations of ATP, Geiselmann et al. (20) were able to detect 2–3 additional ATP binding sites. Similarly, we have observed 5–6 ADP-AlF4− species bound to rho in the presence of RNA (data not shown). Geiselmann et al. noted that the equilibrium dissociation constant of the three weak ATP binding sites (Kd ~ 10 μM) was the same as the enzymatic Km of the ATPase activity (about 10 μM). Furthermore, the three tight sites had a 10-fold tighter Kd value relative to the Km value (20). These findings support the hypothesis that the weak ATP binding sites, those sites that hydrolyze and turn over ATP at a rapid rate, are the catalytic sites. A recent report has shown that binding and hydrolysis of ATP at the catalytic sites is sequential, and only one ATP is hydrolyzed at a fast rate (24). This implies that at any given time there is only one catalytic site that has a tightly bound nucleotide, and ATP binding at the other two catalytic sites is weak, because ATP exchange at those sites is rapid.

Noncatalytic nucleotide binding sites appear to be general to the hexameric helicases, because such sites are now found in at least two hexameric helicases, rho and bacteriophage T7 DNA helicase. Experiments similar to the ones reported in this paper were used to show that the three tight nucleotide binding sites of T7 DNA helicase hexamer are noncatalytic (23). In T7 DNA helicase, these sites contained dTTP and some dTDP, and these nucleotides were exchanged with a rate constant that was much slower than the dTTPase turnover rate. Such sites are well established in the hexameric F1-ATPase protein, which has a high degree of structural and amino acid sequence homology to rho. The noncatalytic sites of the F1-ATPase protein bind ATP, and these ATPs are not hydrolyzed under the conditions of the experiments (28, 29). In the rho protein, the ATPs at the noncatalytic sites are hydrolyzed to ADP and P3, but the hydrolysis products remain tightly bound and their release into solution occurs very slowly. Thus, the noncatalytic sites on the hexameric helicases do show differences from such sites in the F1-ATPase protein. This is understandable because the structures of these proteins are different. Rho and T7 DNA helicases are homohexamers and F1-ATPase is an α3β3 heterohexamer. The α subunits of F1-ATPase are designed to be noncatalytic. That is, the ATP binding site on the α subunit is lined with amino acids that facilitate tight ATP binding, but some critical amino acids that promote efficient hydrolysis of ATP are missing.

In rho, the different rates of ATPase turnovers at the noncatalytic versus the catalytic sites indicate different conformations of these subunits. These conformational changes do not have to be large to be significant. Even small changes in the
position of the amino acids at the active sites can be enough to change the microenvironment of the ATP binding sites and to affect binding and hydrolysis of ATP. Because rho is a homo-hexamer, the asymmetry may be induced upon oligomerization or upon ATP binding/hydrolysis. Based on the similarity of rho with the F1-ATPase protein, we propose that rho hexamer may adopt a C3 or a pseudo-C6 symmetry. Such an intrinsic symmetry (C3 or C3/6) has been proposed by Horiguchi et al. (18) for the rho protein using chemical cross-linking experiments. In the F1-ATPase protein, the two classes of sites, catalytic and noncatalytic, are located on alternating subunits or interfaces between the subunits. This may be the case with the hexameric helicases as well. The exact role of the noncatalytic sites in the hexameric helicases or the F1-ATPase is not clear at present. In helicases, nucleotide binding at these sites may be necessary for the stabilization of the quaternary structure of the helicase, or these sites may play a regulatory function. Alternatively, these subunits may be involved in nucleic acid binding. There are many questions regarding the role of these sites that will need further investigation.

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