Escherichia coli transcription termination protein Rho, an RNA-dependent ATPase, disrupts transcription complexes, releasing RNA and allowing RNA polymerase to recycle. Homohexameric Rho binds three molecules of MgATP in a single class of catalytically competent sites. In rapid mix chemical quench experiments, when Rho saturated with ATP was mixed with RNA and the reaction was quenched after various times, hydrolysis of the three bound ATP molecules was not simultaneous. A hydrolysis burst of one molecule of ATP per hexamer occurred at >300 s⁻¹, followed by steady-state hydrolysis at 30 s⁻¹ per hexamer. This burst also shows that a step following ATP hydrolysis is rate-limiting for overall catalysis and requires communication among the three catalytic sites during net ATP hydrolysis. The rate of hydrolysis of radiolabeled ATP when one labeled and two unlabeled ATP molecules are bound indicates a sequential pattern of hydrolysis. Positive cooperativity of catalysis occurs among the catalytic sites of Rho; when only one ATP molecule is bound per hexamer, ATP hydrolysis upon addition of RNA is 30-fold slower than when ATP is saturating. These behaviors are comparable to those of F₁-type ATPases, with which Rho shares a number of structural features.

*Escherichia coli* transcription termination protein Rho aids in the release of newly synthesized RNA from paused transcription complexes (reviewed in Ref. 1). The homohexameric protein binds nascent RNA and, with the RNA-dependent hydrolysis of ATP, disrupts the ternary transcription complex, releasing product RNA and allowing RNA polymerase to recycle. Homohexameric Rho binds three molecules of MgATP in a single class of catalytically competent sites. In rapid mix chemical quench experiments, when Rho saturated with ATP was mixed with RNA and the reaction was quenched after various times, hydrolysis of the three bound ATP molecules was not simultaneous. A hydrolysis burst of one molecule of ATP per hexamer occurred at >300 s⁻¹, followed by steady-state hydrolysis at 30 s⁻¹ per hexamer. This burst also shows that a step following ATP hydrolysis is rate-limiting for overall catalysis and requires communication among the three catalytic sites during net ATP hydrolysis. The rate of hydrolysis of radiolabeled ATP when one labeled and two unlabeled ATP molecules are bound indicates a sequential pattern of hydrolysis. Positive cooperativity of catalysis occurs among the catalytic sites of Rho; when only one ATP molecule is bound per hexamer, ATP hydrolysis upon addition of RNA is 30-fold slower than when ATP is saturating. These behaviors are comparable to those of F₁-type ATPases, with which Rho shares a number of structural features.

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Catalytic Sites of Escherichia coli Transcription Termination Protein Rho*

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**Escherichia coli** transcription termination protein Rho aids in the release of newly synthesized RNA from paused transcription complexes (reviewed in Ref. 1). The homohexameric protein binds nascent RNA and, with the RNA-dependent hydrolysis of ATP, disrupts the ternary transcription complex, releasing product RNA and allowing RNA polymerase to recycle. The discovery of a 5' → 3' RNA-DNA helicase activity of Rho (2) suggested that Rho might disrupt the RNA-DNA duplex of the transcription bubble. Recent studies of ternary transcription complexes (Refs. 3–5 and reviewed in Ref. 6) suggest that such disruption could be important in transcription termination, as could be the release of the nascent RNA just 5' of the RNA-DNA duplex from its interactions with RNA polymerase. As described by Nudler et al. (7), the interaction of RNA with RNA polymerase immediately 5' from the RNA-DNA hybrid may control the opening and closing of an RNA polymerase clamp around the DNA template near the leading edge of the enzyme, and contribute to the stability of the ternary transcription complex. An appealing model for Rho is one in which the enzyme binds to exposed mRNA behind RNA polymerase and travels 5' → 3' along the RNA as it hydrolyzes ATP, binding and releasing RNA from different parts of the hexamer to accomplish movement (8). Such activity could release nascent RNA from RNA polymerase-binding sites and could constitute the basis for its RNA-DNA helicase activity, both of which might be involved in transcript release from paused ternary transcription complexes. The finding that the same number of ATP molecules per RNA length is hydrolyzed by Rho traveling along RNA and Rho unwinding RNA-DNA hybrids (8) supports this hypothesis.

Rho binds single-stranded RNA, showing preferred entry regions on RNA upstream of eventual transcription termination sites. However, the characteristics of these regions, beyond low secondary structure and some preference for a C-rich, G-poor base composition (9), are too poorly understood to permit their identification by sequence inspection. When bound to RNA, Rho protects 80 bases from ribonuclease degradation (10, 11). The binding of Rho to 10-base RNA oligomers was reported as best fit by three tight and three weaker sites per hexamer (12, 13).

The RNA-dependent hydrolysis of ATP is essential for the transcription termination function of Rho. Two components of ternary transcription complexes, the DNA template and RNA polymerase, are not required to elicit this ATPase activity, thus considerably simplifying study of the reaction (14). The reaction is particularly well stimulated by the RNA homopolymer poly(C), and Rho is frequently assayed *in vitro* by measuring its poly(C)-dependent ATPase activity. Previous work has shown that the Rho hexamer binds three molecules of MgATP in a single class of catalytically competent sites (15, 16). An additional class of three ATP-binding sites of lower affinity has also been suggested (16), although the catalytic activity of these sites was not assessed. The stoichiometry for ATP and RNA oligomer interactions with Rho, together with studies of Rho quaternary structure (17–19), have led to a proposed model of Rho as a trimer of dimers in which adjacent identical subunits may alternate in their ability to bind and hydrolyze ATP and to bind and release RNA (8, 15, 20).

Our goal is to elucidate the molecular mechanism of Rho activity, including the sequence of ATP and RNA binding, ATP hydrolysis, product release, and interactions with other molecules that sum to transcription termination. Travel in a 5' → 3' direction along single-stranded RNA can readily be seen as consistent with the hydrolysis in an ordered sequence of ATP molecules that are bound to Rho, but evidence for such a hydrolysis pattern is lacking. We present the results of rapid mix chemical quench experiments and isotope partitioning studies, which show that hydrolysis of the three bound ATP
molecules is sequential. These results also indicate communication among the active sites of Rho. In addition, we show catalytic cooperativity among Rho active sites.

**MATERIALS AND METHODS**

**Enzymes, Substrates, and Buffers—** Wild-type Rho from *E. coli* was purified as described (21) from strain AR120/A6 containing plasmid p9ASE (22). The concentration of Rho was spectrophotometrically determined by the equation:

\[
\text{Abs} = 2.25 \text{ cm}^{-1} \text{ mg}^{-1}
\]

The enzyme preparation used for most experiments had a specific activity with poly(C) at 37 °C of 12–15 units mg\(^{-1}\). Some experiments were repeated with an independent enzyme preparation that had a specific activity of 22–26 units mg\(^{-1}\). Polyacrylamide gels. A unit of activity is that amount of enzyme that catalyzes a reaction with a 100-fold higher than normal concentrations of both Rho and poly(U) and at low ATP levels. The concentrations of reactants in the mixer in most experiments as follows: Rho, 0.88 mg/ml (10 μM active sites); \([γ-32P]ATP or its analogs, 0.1 m M (the equivalent of 30 ATP molecules per Rho hexamer); RNA, 0.2 mg/ml (600 μM bases); thus two 100-base lengths of RNA per hexamer. These values were chosen so that the ATP concentration was sufficiently high that its on-rate (estimated from \(k_{on}/K_a\)) was not limiting, and the enzyme concentration was such that turnover of a single ATP per hexamer would produce product \(32P\) significantly above the background level of \(32P\) in the nucleoside triphosphate substrates. In experiments with \(\gamma-32P\)ATP following charcoal absorption of adenine nucleotides as described above.

The rate of ATP hydrolysis by Rho in the absence of RNA was measured using Rho at 1.76 μg/ml in TAGME buffer with 200 μM \([γ-32P]ATP at 20,000 cpn\)/ml. At \(t = 0\) and at 30–60 min intervals to \(t = 2\) h, the amount of \(32P\) was determined in 50-μl samples following charcoal absorption of adenine nucleotides (described below).

To assay for a burst of ATP hydrolysis in the absence of RNA, 90–100 μl mixtures were prepared, each containing 10 μM \([γ-32P]ATP at 660,000 cpn\)/ml (with 3.3% background \(32P\) radioactivity) or 0.5 mM \([γ-32P]TNP-ATP at 1500 cpn\)/ml (0.5% background \(32P\) counts). The final concentrations of reactants used for most experiments were 0.25 μM Rho, 100 μM poly(C), and 300 μg/ml in poly(U), and at low ATP levels.

**Enzymes, Substrates, and Buffers—**

**Isotope Partitioning—** These experiments, using 0.35 μM Rho hexamer, 0.17–6.0 μM \([γ-32P]ATP at 511 cpn\)/ml, were carried out as described in Stitt (15), except that in most cases TAGME buffer was used, and a 50-μl sample was injected into 150 μl of chase solution, followed by 50 μl of 50% w/v trichloroacetic acid as quenching agent. ATP hydrolysis was monitored as above by measuring the production of \(32P\) from \([γ-32P]ATP following charcoal precipitation of adenine nucleotides. These same binding mixtures were used for both binding and isotope partitioning measurements. In experiments with \([γ-32P]TNP-ATP, the ligand was 0.19–5.9 μg/ml at 1075 cpn\)/ml.

\(\text{LO} \text{ Exchange Experiments—}\) 0.5 ml reactions were prepared in a final concentration of 80% \([\text{IO} ]\) in TAGME buffer; several different protocols were used as follows. 1) For \(V_{\text{max}}\) conditions, the reaction contained 1 μg of Rho, 0.4 μg of poly(C), and 2 μM ATP, 3 μM in magnesium acetate. This reaction was quenched after 30 min at 37 °C, when about 60% of the ATP had been hydrolyzed, by addition of EDTA to 10 mM. As a control, an identical mixture lacking Rho was also prepared. 2) A reaction at a low ATP concentration of 5 μM was prepared similarly to the \(V_{\text{max}}\) reaction, with the addition of an ATP-regenerating system consisting of phosphoenolpyruvate at 1 mM plus 5 μg of pyruvate kinase. This reaction was incubated at 37 °C for 3 hr. In this case, the reaction was quenched after 5 min at 37 °C. In 0.5 ml reactions of a similar design, 2 μM ATP hydrolysis, a mixture similar to the first was prepared, with ATP replaced by 2 m M ADP plus 2 m M Pi. This mixture was incubated at 37 °C for 5.5 hr to addition of the EDTA quench. Following quenching, each reaction mixture was filtered through an Amicon Microcon 10 ultrafiltration apparatus and was stored at −20 °C until analysis.

At this time the samples were thawed, supplemented with D2O to 10% v/v, and free nucleotide were corrected for this adventitious binding. For binding experiments, a Microcon-10 ultrafiltration apparatus was used. In control samples from which Rho was omitted, 3–5% of the binding was observed. For experiments with \(\gamma-32P\)ATP, the assay was performed with 5-fold more Rho than usual (2.5 rather than 0.5 μg/ml in the assay) and for 40 min (rather than the standard 20 min at room temperature), assays involving poly(U) and ATP used 10-fold higher than the normal concentrations of both Rho and poly(U) and were for 40 min; for TNP-ATP with poly(U) and ATP with poly(A), final concentrations of 1.33 mg/ml Rho and 300 μg/ml polyadenylate were used, with multiple P determinations. In these cases, similar reaction mixtures lacking either enzyme or RNA were served as controls.

**ATP Hydrolysis by Rho in the Absence of RNA—** The rate of ATP hydrolysis by Rho in the absence of RNA was measured using Rho at 1.76 μg/ml in TAGME buffer with 200 μM \([γ-32P]ATP at 20,000 cpn\)/ml. At \(t = 0\) and at 30–60 min intervals to \(t = 2\) h, the amount of \(32P\) was determined in 50-μl samples following charcoal absorption of adenine nucleotides (described below).

The abbreviations used are: ATP-SS, adenosine 5′-(γ-thio)triphosphate; TNP-ATP, TNP-ADP, the 2′,3′-O-(2,4,6-trinitrophenyl) derivatives of ATP and ADP.
that to explain the isotope partitioning results, ATP molecules are hydrolyzed because of a faster off-rate. (The buffer all bound ATP molecules are hydrolyzed upon addition of isotope partitioning experiments: in chloride-containing glutamate-containing buffer. This difference is also seen versus types of Rho is the two types of Rho using poly(C) as the RNA cofactor; values for published earlier (15).) Table I shows data obtained at 22 °C for the of adenine nucleotide interactions with RhoE155K was published earlier (15). Table I shows data obtained at 22 °C for the Rho between the RNA-binding amino terminus and the ATP-binding domain (27). Parameters important to the present work were therefore remeasured for the true wild type Rho protein used in an earlier determination of kinetic mechanism (15) is now known to carry the mutation (RhoE155K (22, 26); this mutation lies in a proposed hinge region of the poly(C)-stimulated Rho data in TKME are at 37 °C (15).

and analyzed by one-dimensional $^{31}$P NMR with proton decoupling using a Bruker DRX300 spectrometer operating at 121.5 MHz.

**RESULTS**

**Wild Type Rho Compared with RhoE155K**

The *E. coli* Rho protein used in an earlier determination of the kinetic mechanism (15) is now known to carry the mutation E155K (22, 26); this mutation lies in a proposed hinge region of Rho between the RNA-binding amino terminus and the ATP-binding domain (27). Parameters important to the present work were therefore remeasured for the true wild type Rho (Rho+) that was used in these studies. (A more complete study of adenine nucleotide interactions with RhoE155K was published earlier (15).) Table I shows data obtained at 22 °C for the two types of Rho using poly(C) as the RNA cofactor; values for $k_{cat}$, $K_m$, $K_D$, and $n$ values for binding and isotope partitioning are unaffected by the mutation. Rho+ and RhoE155K also have similar $K_m$ values at 37 °C for ATP, 8–10 μM, and similar hexamer $V_{max}$ values with poly(C). A notable feature of both types of Rho is the ~10-fold tighter binding of ATP in chloride-versus glutamate-containing buffer. This difference is also seen in isotope partitioning experiments: in chloride-containing buffer all bound ATP molecules are hydrolyzed upon addition of RNA, but in glutamate-containing buffer only ~70% of bound ATP molecules are hydrolyzed because of a faster off-rate. (The off-rates in Table II are calculated, minimum values; we note that to explain the isotope partitioning results, $k_{off}$ in glutamate buffer must be slightly faster than the calculated 6.6 s$^{-1}$.)

For both types of Rho, our experiments indicated 3 ATP molecules bound per hexamer (Fig. 1) and did not show a second class of binding sites with lower affinity for ATP (16). If data from ultrafiltration binding experiments were not corrected for ATP bound to the apparatus in the absence of Rho, then, as shown in the inset to Fig. 1, an additional class of ATP-binding sites appears to exist. These “sites” apparently represent adventitious binding of ATP to the apparatus, rather than a second class of sites on Rho. Earlier work demonstrated three adenine nucleotide-binding sites on Rho both in the absence and presence of RNA (15).

**Very Slow ATP Hydrolysis by Rho without RNA**

Previously (15), the rate of ATP hydrolysis by RhoE155K at 37 °C in the absence of RNA was estimated to be no greater than 2 ATP molecules/hexamer/h. Additional measurements showed that the rate at 22 °C for Rho+ is linear, with values from 1.5 to $4.4 \times 10^{-4}$ s$^{-1}$ hexamer$^{-1}$, 100,000-fold slower than the poly(C)-stimulated $V_{max}$ (data not shown). In addition,
mix apparatus. See “Materials and Methods” for details. Rho + ATP in one syringe were mixed with poly(C) from the second syringe; the reaction was quenched after the indicated times, and the amount of ATP hydrolysis was measured (see “Materials and Methods”). Data were corrected for ATP hydrolysis by Rho in the syringe in the absence of RNA and were fit to a line by the least squares method.

Rather, they indicate that the rate of hydrolysis of one of the 3 ATP molecules bound to Rho is hydrolyzed faster than the rest. ATPase with poly(C) is only 4.5 s⁻¹ per hexamer. The results were similar to those at 22 °C: a burst at the earliest time measured after mixing, followed by slower steady-state hydrolysis of the remaining ATP molecules (Table II; data not shown).

Hydrolysis of the 3 ATP molecules bound to Rho is thus not simultaneous. The results also indicate that the three active sites are not independent; communication among them is required to explain how catalysis at two of the three sites is delayed. Before hydrolysis of a second bound ATP molecule occurs, some slow process with a net rate equivalent to the steady-state $k_{cat}$ is essential. What is this process? It could be that the release of one of the products, ADP, $P_i$, or RNA, is essential and slow and must precede hydrolysis at additional sites or that an enzyme conformation change following ATP hydrolysis is rate-limiting. (Under the conditions used, RNA and ATP binding are fast.) To try to identify the slow process, experiments were performed using ATP analogs or alternative RNA cofactors.

Alternative Substrates In Rapid Mix Experiments

TNP-ATP Behaves Like ATP—An ATP analog that is slowly hydrolyzed by Rho is the ribose-modified nucleotide TNP-ATP: $V_{max}$ at 22 °C with poly(C) was 1 unit mg⁻¹, compared with 4 units mg⁻¹ for ATP. Like ATP, TNP-ATP is not hydrolyzed by Rho at a significant rate until RNA binds (data not shown; see “Materials and Methods”). In rapid mix chemical quench experiments, also like ATP, 0.4–0.5 mol of TNP-ATP per mol hexamer was found to be hydrolyzed at the earliest time measured (Table II; data not shown). These data suggest, first, that the release of $P_i$ from Rho may not be the rate-limiting step, since $P_i$ is a common product of ATP and TNP-ATP hydrolyses and, second, that the release of the nucleoside diphosphate may be the slow step of steady-state catalysis.

Poly(U) Behaves Similarly to Poly(C) —When the homopolymer poly(U) was substituted for poly(C), the ATPase reaction of Rho at 22 °C had a steady-state hexamer $V_{max}$ of 0.25 ± 0.1 units per s⁻¹ instead of 30 s⁻¹. In rapid mix chemical quench experiments, a burst of about one ATP molecule per hexamer was found at the shortest time measured (Table II). In contrast to experiments with ATP, these results suggest that RNA release from Rho may be rate-limiting.

Burst Kinetics with TNP-ATP and Poly(U)—A rapid mix experiment in which TNP-ATP was substituted for ATP and
See "Materials and Methods" for details. Rho in one syringe was mixed with TNP-ATP + poly(U) from the second syringe; the reaction was quenched after the indicated times, and the amount of ally found to slow the chemistry of phosphoryl transfers (29). The high standard error reflects the complexity of the fit.

Table II. (The rapid mix apparatus, See "Materials and Methods" for details. Rho in one syringe was mixed with TNP-ATP + poly(U) from the second syringe; the reaction was quenched after the indicated times, and the amount of ally found to slow the chemistry of phosphoryl transfers (29). The high standard error reflects the complexity of the fit.)

Table III. Hexamer $V_{max}$ values at 22 °C (nucleotides s$^{-1}$)

|        | Poly(C) | Poly(U) | Poly(A) |
|--------|---------|---------|---------|
| Poly(C) | 30 $\pm$ 2 | 6.3 $\pm$ 0.3 | |
| Poly(U) | 0.25 $\pm$ 0.1 | 0.025 $\pm$ 0.025 | |
| Poly(A) | 0.021 $\pm$ 0.007 | 0.0011$^a$ | |

$^a$ Rate was determined by linear least squares fit to a 7-point line with r.m.s. = 0.97.

Sequential Hydrolysis of Bound ATP Molecules Is Also Suggested by Isotope Partitioning Results

The technique of isotope partitioning (32, 33) allows the determination of the relative rates of productive versus dissociative fates for enzyme-bound substrates. In these experiments, samples of a mixture of Rho plus [$\gamma$-32P]ATP were injected into a rapidly stirred "chase" solution containing the following: 1) poly(C) to complete the requirements for enzymatic ATP hydrolysis, and 2) an excess of nonradioactive ATP. Labeled ATP that is bound to the enzyme at the time of its injection into the chase may either be hydrolyzed or dissociate from the enzyme and be prevented from rebinding by dilution in the nonradioactive ATP. The hydrolysis of labeled ATP is monitored by measuring the production of $^{32}$P$_i$. For E155K Rho, for example, at 37 °C in chloride-containing buffer the partitioning of bound ATP is completely toward $P_i$ product, demonstrating a slow off-rate for bound ATP relative to forward catalysis (15). Table I gives the values obtained in isotope partitioning experiments at room temperature in which the enzyme was initially saturated with radiolabeled ATP. In glutamate-containing buffer, only ~70% of the initially bound ATP molecules were hydrolyzed. Similar results were found when the Rho concentration was 3.3 μM hexamer, as was used in rapid mix experiments (data not shown). These results support the conclusion that hydrolysis at some active sites is delayed, and the delay is sufficiently long that a portion of the bound substrate dissociates and is not hydrolyzed. The situation is more extreme when TNP-ATP is the substrate. ATP binding data indicate three equivalent binding sites with off-rates, calculated using the measured $K_D$ and $k_{cat}/K_m$ as the on-rate, of 6.6 s$^{-1}$. For TNP-ATP, a similar calculation yields an off-rate of 14 s$^{-1}$. In isotope partitioning experiments with [$\gamma$-32P]TNP-ATP, although a maximum of at least 3 molecules of TNP-ATP bound to Rho, only one (the burst site) was hydrolyzed (data not shown).

Various hydrolysis patterns can be proposed to explain the observed isotope partitioning results. In all cases considered here, unlabeled ATP is assumed to bind to catalytic sites following hydrolysis of labeled ATP and release of products. 1) In a sequential ordered model, hydrolysis could occur in a sequential and ordered fashion around the hexamer, with complete hydrolysis at the burst site (site 1), predominant hydrolysis of the next ATP (site 2), and less complete hydrolysis of the final ATP (site 3) because of dissociation. In this model the pattern of site firing would be fixed, 1–2–3–1–2–3– etc., and the apparent hydrolysis rate constant for each of the three initial bound, labeled ATP molecules would be successively smaller. 2) In a random sequential model, ATP hydrolysis could occur sequentially but with a random pattern of site firing, for example 1–2–1–3–2–1–3– etc. In this case, the two labeled ATP molecules remaining following the burst would be hydrolyzed more slowly than if the pattern were sequential and ordered. Hydrolysis of the two labeled ATP molecules would require multiple catalytic turnovers, with a decreasing proportion of the labeled ATP hydrolyzed in each successive turnover. 3) In an independent model, a more complex hydrolysis pattern is envisioned, in which a burst at one site is followed by independent hydrolysis
Rapid-Mix-with-Chase Experiments Eliminate Random Sequential Hydrolysis Pattern

To discriminate among these three models for the pattern of ATP hydrolysis, we carried out rapid mix experiments in which Rho plus sufficient [γ-32P]ATP to fill its three active sites was mixed with RNA plus excess unlabeled ATP, and the mixture was quenched after various times to follow the hydrolysis rate of the radio labeled substrates. The unlabelled ATP dilutes dissociated radioactive ATP and greatly slows its hydrolysis. The results of one experiment are shown in Fig. 5, as are theoretical predictions for a hydrolysis burst of one ATP followed by random sequential, sequential ordered, or independent hydrolysis patterns of the remaining two labeled ATP molecules. These results clearly eliminate the random sequential model, since the predicted points are far from those observed.

Rapid Mix Results with Substoichiometric ATP Show ATP-Dependent Communication among Active Sites

To discriminate between sequential ordered and independent models, we performed rapid mix experiments in which RNA was mixed with Rho containing only a single bound [γ-32P]ATP molecule per hexamer. Surprisingly, when Rho with an average of either 0.13 or 0.6 mol of [γ-32P]ATP bound per mol hexamer was mixed with poly(C), no burst of [γ-32P]ATP hydrolysis was seen, and hydrolysis proceeded at a single-site

Materials and Methods

Rapid mix experiments in which Rho saturated with [γ-32P]ATP was mixed with RNA + excess unlabeled ATP. See “Materials and Methods” for details. 6 μM Rho hexamer in 33 μM [γ-32P]ATP in one syringe were mixed with poly(C) in 20 mM ATP from the second syringe; the reactions were quenched after the indicated times, and the amount of 32P was measured. Data (filled circles) were corrected for [γ-32P]ATP hydrolysis by Rho in the syringe in the absence of RNA. Theoretical curves have been drawn for the expected amounts of ATP hydrolyzed versus time for these different hydrolysis patterns. Dotted line, burst of one molecule of [γ-32P]ATP/hexamer followed by sequential, fixed order hydrolysis of the remaining two labeled ATP molecules, y = 1 + A(1 − e−kt) + B(1 − e−kt), A corresponds to the burst site ATP, and A and B are proportions of ATP in each of the other two active sites that are hydrolyzed (as opposed to dissociating). For site 2, k1 = 17 s⁻¹, the Vmax hydrolysis rate; for site 3, k2 = 8.5 s⁻¹, half Vmax (because catalysis at this site is delayed until catalysis at the second site is complete). Using an off-rate of 6.6 s⁻¹, estimated from Kd and a minimal on-rate for ATP based on kcat/Km, A = 0.72 and B = 0.56. Solid line, burst of one molecule of [γ-32P]ATP/hexamer followed by independent hydrolysis of the remaining two labeled ATP molecules, y = 1 + 5.4(1 − e−kt), 1 corresponds to the burst site ATP; 1.5 is the total additional amount of labeled ATP that was hydrolyzed in the experiment, and k = 17 s⁻¹. Dashed line, burst of one molecule of [γ-32P]ATP/hexamer followed by sequential, random order hydrolysis of the remaining two labeled ATP molecules. The amount of labeled ATP in sites 2 + 3 that is hydrolyzed after n active sites have fired is calculated from the proportion that partitions toward hydrolysis, (kcat/n)(kcat/n) + koff, multiplied by the proportion of sites with labeled ATP bound, in this case, (2/3)n. The total amount of labeled ATP that has been hydrolyzed after n catalytic site turnovers is thus

\[ y = 1 + \sum_{n=2}^{\infty} \left( \frac{k_{\text{cat}}}{n-1} + k_{\text{off}} \right) \left( \frac{2}{3} \right)^{n-1} \]  

(Eq. 2)

where 1 is the burst site ATP.
rate of only 0.3–0.4 s⁻¹ (Fig. 6; panel A and open symbols of panel B), 30-fold slower than when ATP was saturating (Table II). These unexpected results reveal a previously unknown feature of Rho: a requirement for ATP binding in more than one of the active sites for significant rates of ATP hydrolysis. This evidence demonstrates a new feature of interaction among the catalytic sites of Rho, different from the interaction required to explain non-simultaneous hydrolysis of bound ATP molecules.

To confirm that the labeled ATP in the above experiments was properly bound in active catalytic sites of Rho, the experiment was repeated with the addition of a high concentration of nonradioactive ATP to the poly(C)-containing syringe of the rapid mix apparatus (this experiment is termed “low labeled ATP with chase”). Upon addition of RNA + unlabeled ATP, hydrolysis was much faster (Fig. 6B, filled symbols), with 60–70% of the substoichiometric radioactive ATP bound to Rho hydrolyzed by less than 400 ms after mixing. Binding experiments showed that the unhydrolyzed [γ-³²P]ATP under these conditions had dissociated from Rho (data not shown). The results are consistent with those from manual isotope partitioning experiments at 22 °C in the same buffer (see above), in which one-third of the bound ATP undergoes dissociation from the enzyme rather than hydrolysis, and show that the radiolabeled ATP was productively bound to Rho.

In the low labeled ATP with chase experiments just described, the high concentration of nonradioactive ATP (20 mM) that is introduced simultaneously with the RNA will bind to Rho extremely rapidly; >33,000 s⁻¹ using k₉/Kᵥ, as an estimate of Kᵥ. Thus all vacant active sites will likely fill with nonradioactive ATP before significant [γ-³²P]ATP dissociates at ≈6.6 s⁻¹. The rate of RNA binding to the hexamer will determine whether RNA binds before, during, or after the vacant ATP sites have been filled. Once a long RNA molecule has bound in one site of Rho, filling of other RNA sites on the same molecule by other portions of the same RNA molecule is envisioned as a rapid intramolecular process. Thus the on-rate for RNA may be approximated by the rate of the initial productive interaction. The most rapid RNA binding would occur if the RNA on-rate were as fast as the small molecule diffusion limit of 10⁷–10¹⁰ M⁻¹ s⁻¹ and interaction with one base of the RNA polymer were sufficient for binding. In this (unlikely) case, the maximal RNA binding rate under the experimental conditions would be 10³–10⁴ s⁻¹, slower than that of ATP binding (>33,000 s⁻¹). Thus low labeled ATP with chase experiments follow the hydrolysis of initially bound, labeled ATP (not more than one per hexamer) upon RNA binding to Rho. By the time RNA binds, all unliganded Rho active sites have been filled with unlabeled ATP.

Several patterns of labeled ATP hydrolysis could occur in the low labeled ATP with chase experiment. 1) If the first active unit of Rho that binds ATP is somehow recognizable and is consequently also the first to bind RNA, then all of the labeled ATP will be hydrolyzed with burst kinetics, since labeled ATP was the first to bind. 2) Similarly, if the last active unit that binds ATP is the first to bind RNA, then the burst will be exclusively of unlabeled ATP and hence will not be detected. 3) Random binding of RNA to Rho should produce an initial burst comprising one-third of the bound labeled ATP, with hydrolysis of the remaining bound, labeled ATP dependent on its partitioning between catalysis and dissociation at each site. If the three active sites fire sequentially, then the burst will be followed by two successively slower (and smaller, as ATP dissociates) rate phases. If hydrolysis in the two remaining sites is independent, the burst will be followed by a single catalytic rate. Fig. 7 shows computer-generated fits to data from low labeled ATP with chase experiments. Clearly a single exponential phase, either with (Fig. 7A) or without a burst (Fig. 7B), fits the data more closely, both with (Fig. 7E) or without a burst (Fig. 7D). The two rate phases can also be seen as curvature in the plot of the low labeled ATP with chase results in Fig. 6B (filled squares), which are from experiments carried out with independent Rho and [γ-³²P]ATP preparations. We therefore conclude that the sequential ordered ATP hydrolysis pattern is the correct model.

Interestingly, in these experiments we consistently found the size of the burst to be less than 20% of the labeled ATP rather than the expected 33%. As described above, such a result could be interpreted as a bias in RNA binding to Rho, against its binding to the site that bound ATP first (which, in these experiments, contained labeled ATP).

In these experiments with Rho plus a low concentration of [γ-³²P]ATP in one rapid mix syringe, only a minor correction of the data was necessary for the hydrolysis of ATP by Rho in the absence of RNA. This rate, at substoichiometric ATP, was 3 ×
10⁻⁶ s⁻¹, approximately 100-fold slower than the rate when all ATP-binding sites were filled. Thus, in the absence of RNA as well as in its presence, the hydrolysis of ATP by Rho is slowed when ATP is not saturating.

**DISCUSSION**

Rapid mix chemical quench and isotope partitioning data developed here support several important conclusions concerning the Rho catalytic mechanism. First, upon RNA binding, hydrolysis of the three molecules of ATP bound per Rho hexamer is sequential and occurs in a fixed order. This hydrolysis pattern requires communication and asymmetry among the three active sites during net catalysis. Second, $V_{\text{max}}$ catalytic rates with different nucleotide substrates and RNA cofactors suggest that the slow step of catalysis is an enzyme conformation change. Finally, ATP bound in more than one active site of Rho is required for $V_{\text{max}}$ catalysis, indicating catalytic cooperativity among the active sites.

**Sequential Hydrolysis of 3 ATP Molecules Bound to Rho Requires Catalytic Site Interaction and Asymmetry of the Rho Hexamer**—Upon RNA binding, Rho rapidly hydrolyzes one of the three molecules of bound ATP (burst kinetics) and then sequentially hydrolyzes the remaining two ATP molecules at successively slower rates (Figs. 2, 5, 6B, and 7). Since all components necessary for catalysis are present on the enzyme, communication among the active sites is required to explain why the three bound ATP molecules are not hydrolyzed simultaneously. The two competent catalytic sites that do not rapidly hydrolyze their bound ATP molecules when RNA is encountered must differ in some way from the burst site.

One possible explanation is that the difference among the active sites could preexist in the Rho hexamer. Evidence for possible preexisting asymmetry in the unliganded Rho hexamer arises from the “notched” or “lockwasher” appearance in electron micrographs of some Rho hexamers (11, 34–36). Such asymmetry could persist after RNA binding.

A second possibility is that asymmetry in the hexamer is not preexistent but is induced by RNA binding; RNA bound to Rho is thought to extend around the outside of the enzyme (17), so RNA must associate with one active unit from which its 5’ end protrudes, be internally in contact with a second unit, and extend its 3’ end from a third (20). These features may be sufficient to distinguish the three active units.

In conflict with the above analysis are the results of nucleotide binding experiments, which give no indication of asymmetry, either in the presence or absence of RNA (Fig. 1; Refs. 15 and 16). A difference in binding of only 1.4 kcal, the equivalent of one hydrogen bond, would be expected to lead to an easily measured 10-fold difference in $K_p$.

Finally, ATP hydrolysis to ADP + Pᵢ at the burst site could induce conformation changes in one or more subunits that lead to asymmetry. This model is consistent with the nucleotide binding data and is unlike the previous two models in that any of the three active units could be the burst site. The other two models for asymmetry suggest a particular location for the first ATP hydrolysis within the hexamer, such as at one end of the lockwasher or at the position closest to an RNA exit point. At present, no firm explanation of the source of asymmetry in Rho is available.

**The Slow Step of Steady-state Catalysis Is an Enzyme Conformation Change**—In principle, steady-state hydrolysis rates could be determined by nucleoside triphosphate or polynucleotide on-rates, product off-rates, or an enzyme conformational change. We discuss each of these possibilities in turn. With respect to substrate on-rates, under the experimental conditions used here, on-rates are not limiting. For example, a minimal estimate of the on-rate for ATP is obtained from $k_{\text{cat}}/K_m = 3.33 \times 10^6 \text{M}^{-1}\text{s}^{-1}$. At the 100 μM level of ATP used in many of the rapid mix experiments, the on-rate is thus 330 s⁻¹, far faster than the active unit catalysis rate (10 s⁻¹). With respect to product off-rates, the off-rate for Pᵢ release is not likely to be rate-limiting, because Pᵢ is a common element in the hydrolyses of TNP-ATP and ATP, which show different $V_{\text{max}}$ values. The $V_{\text{max}}$ for TNP-ATP might be lower because the TNP-ADP product is released more slowly from the enzyme than is ADP. However, the results of isotope partitioning experiments and an estimate of $k_{\text{off}}$ based on the measured $K_p$ and using $k_{\text{cat}}/K_m$ for the on-rate gives an off-rate for TNP-ATP faster than that for ATP. Similarly, Galluppi and Richardson (10) found a faster off-rate for poly(U), and a 10-fold higher $K_p$ for poly(U) than for poly(C) has also been reported (37, 38). However, there may be two types of RNA-binding sites on Rho, one involved in stabilization of Rho-RNA complexes and one involved in ATPase activation (13, 39), and it is unclear whether the results just cited pertain to the site(s) involved in ATP hydrolysis. Perhaps of greater significance is the report of a 30-fold higher $K_p$ during ATP hydrolysis for the oligomer U₇ compared with C₇, under conditions where these RNA oligomers must be binding in the sites that are relevant for catalysis (39). We have confirmed the latter observation using U₁₀ and C₁₀ (38). These results suggest that slow polynucleotide off-rates from Rho when homopolymers other than poly(C) are used are not responsible for slowing catalysis.

Experiments involving simultaneous use of alternative RNA cofactors and nucleotide substrates showed far greater effects on $V_{\text{max}}$ than did experiments involving either substitution singly (Table III), indicating that these two types of ligand probably affect the same catalytic step. An enzyme conformation change that occurs when both types of ligand are bound is a good candidate for the rate-limiting step. Since the chemistry of ATP hydrolysis by Rho is fast, the rate-limiting enzyme conformation change must occur when both RNA and the hydrolysis product ADP are bound. Several conformation changes of Rho have been inferred to result from ligand binding, as detected by the trypsin sensitivity of Rho ATPase activity and the appearance of tryptic cleavage products. The trypsin sensitivity of Rho has been found to be enhanced by RNA binding (40, 41). ATP or ADP binding, on the other hand, protects a cleavage site at Arg-128, between the RNA-binding and ATP-binding domains (40). Thus there is evidence for protein conformation changes that result from events that occur during catalysis. Our data suggest that one such change, which takes place when both NDP and RNA are bound, is the rate-limiting step of the catalytic cycle.

**Requirement for More Than One ATP/Hexamer for $V_{\text{max}}$ Catalysis**—The finding that ATP bound in more than one catalytic site is required for very rapid ATP hydrolysis at one site (burst kinetics) and for subsequent catalysis at $V_{\text{max}}$ (Fig. 6) also requires interaction among Rho active sites distinct from the active site communication during $V_{\text{max}}$ hydrolysis that results in sequential hydrolysis of the three bound ATP molecules. It is strongly reminiscent of the catalytic site cooperativity of mitochondrial Fᵣ-ATPase, in which ATP bound in a single catalytic site is slowly hydrolyzed by the enzyme without release of products until ATP is bound in a second catalytic site (42–43). In the case of Rho, ATP bound in a catalytic site is not efficiently hydrolyzed unless two conditions are met as follows: 1) additional ATP is bound in catalytic site(s) on the hexamer, and 2) RNA is bound.

**A Model for Catalysis**—Our model for the hydrolysis of three labeled ATP molecules bound to Rho when RNA and excess unlabeled ATP are added is shown in Scheme 1. Rho is drawn as a trimer of active units, with unspecified RNA interactions.
ATP bound to one active unit of Rho (the burst site) is hydrolyzed rapidly upon RNA binding. Products are then released from this site, and a new ATP binds. ATP bound in a second Rho active unit, different from the burst site, is hydrolyzed next. Following product release and binding of a new ATP at this site, ATP remaining in the third active unit is hydrolyzed, products are released, and a new ATP binds. The proportion of nucleotide in each active site that is hydrolyzed is determined by the nucleotide off-rate and the rate of forward catalysis. In Scheme 1, the size and thickness of the type face vary to indicate relative amounts of nucleotides under our experimental conditions. Scheme 1 indicates an average of 3 molecules of ATP bound per Rho hexamer during steady-state catalysis; our present data on catalytic site cooperativity are consistent with an average of either 2 or 3 ATP molecules bound. Although we have not presented any data here concerning RNA interactions with Rho, it might be expected that the order in which the active units fire is determined by the $5' \rightarrow 3'$ asymmetry of the bound RNA.

Similarity with Other Systems—Rho constitutes the third system in which evidence now supports sequential NTP hydrolysis by an essentially hexameric protein in three catalytic sites in a fixed order; the other two systems are F$_1$-ATPase (44) and T7 4A' helicase (45). Dombroski and Platt (46) and Opperman and Richardson (27) pointed out amino acid sequence similarities between Rho and other ATP-binding proteins, including F$_1$, and Miwa et al. (47) used the crystal structure of F$_1$ as a basis for modeling the structure of the ATP-binding portion of Rho. The present work extends the similarities with F$_1$ beyond structural aspects to the catalytic pattern (active sites fire sequentially in a fixed order) and the existence of catalytic cooperativity (events in the catalytic cycle in one active site are required for efficient completion of the catalytic cycle at another active site).

A feature common to F$_1$ and the T7 helicase is key asymmetric interactions involving the central region of the hexamer. In F$_1$ there is rotation of the central y subunit within the $\alpha_y\beta_3$ hexamer (48, 49), and for T7 4A' helicase DNA binding is to one or two subunits of the hexamer within the central region (50, 51). A similar situation has been proposed for Rho (19, 47, 52); in a type of tethered tracking model for termination (53, 54), tight binding sites may hold RNA relatively immobile around the outside of the Rho hexamer, whereas ATP hydrolysis activity is coordinated with looser RNA binding and release in central sites. The net result is feeding of the $3'$ end of the RNA through the center of Rho as the protein moves $5'$ to $3'$ along the RNA (19, 52). This type of model could provide the asymmetry required by our present results.

Catalytic Cooperativity Is Consistent with Inactivation

Data—The current data confirm and help to explain the results of previous enzyme inactivation experiments that employed ATP analogs, in which the stoichiometry of inactivation was found to be 1 mol of inactivator per hexamer (55, 56). Interaction among the three active sites, required for $V_{max}$ catalysis, means that inactivation of one active site should prevent all sites from functioning. An enzyme molecule with one inactive catalytic unit might be able to hydrolyze a single ATP at one or both unmodified active sites but be incapable of further catalysis, or it might be completely inactive.

Ligand-binding Sites on Rho—Although both molecular genetics and electrospray mass spectrometry measurements indicate that Rho has six identical subunits (57), only three ATP-binding sites are detected. As suggested by Stitt (15), these results may indicate that ATP binding to some subunits of Rho prevents ATP binding to other subunits at the same time or may indicate pre-existing asymmetry of the enzyme such that only 3 ATP molecules can bind simultaneously. The results also suggest that the six subunits could alternate in their ability to bind and hydrolyze ATP and bind and release RNA. In such a model, Rho resembles the dimeric Rep DNA helicase and the hexameric T7 4A' helicase, where different affinities for DNA depend on whether NTP or NDP is bound (58, 59).

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