ADP but Not P_i Dissociation Contributes to Rate Limitation for Escherichia coli Rho*

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To define the molecular mechanism by which ATP hydrolysis powers the 5'→3' travel of homohexameric Escherichia coli transcription termination factor Rho along RNA, rates for association and dissociation of non-RNA substrates and products were measured. Rapid mix/chemical quench and stopped-flow spectrofluorometry measurements were carried out with Rho and [γ-32P]ATP, mantADP, or fluorescently tagged E. coli phosphate-binding protein. The results indicate that the P_i off-rate is not rate limiting, but at ~90 s⁻¹, the ADP dissociation rate is comparable to the 30 s⁻¹ k_cat. Previous results indicate that the chemistry step of ATP hydrolysis by Rho is at least 10-fold faster than the overall catalytic cycle. The as yet unmeasured RNA dissociation step, which could be associated with a protein conformation change, might also be a rate-limiting factor.

Escherichia coli transcription termination protein Rho is a homohexameric RNA/DNA helicase that uses the energy from ATP hydrolysis to travel directionally 5'→3' along nascent RNA and to disrupt the ternary transcription complex when it reaches transcribing RNA polymerase (for reviews, see Refs. 1, 2). The release of RNA from the DNA template and from RNA polymerase is thought to result from Rho travel along RNA, a process fueled by RNA-dependent ATP hydrolysis by Rho. This energy expenditure presumably leads to protein conformational changes that allow the repeated binding and release of RNA by Rho that constitute travel. The coordination of ATP hydrolysis with Rho/RNA interactions and the molecular details of these processes are not yet clear.

To achieve transcript release, Rho binds an 80-base stretch of the relevant RNA (3, 4). This is accomplished by RNA binding on one face of Rho along its six circularly arranged subunits (5) and then passing through a central hole in the hexamer (6). Neither the subsequent movements of RNA in response to ATP hydrolysis nor the manner of final RNA release from Rho are known.

The directional movement of Rho with respect to RNA is likely to involve travel of the RNA through the central hole of the Rho hexamer. Rho is thus a specific example of a system in which a multisubunit protein moves an unfolded macromolecule (nucleic acid or protein); examples include not only ATP-dependent helicases such as Rho, but also ATP-dependent poly-Ases (7) and virus packaging proteins (8). Although there are proposed mecha nochemical models for several of these systems, proof for any is incomplete. Protein structures underlie current mechanism proposals: connecting actions are predicted when different structures with bound substrates or products are available (e.g. 8). Knowledge of the order and kinetics of all steps of the catalytic cycle, however, is indispensable for mechanistic understanding.

It has previously been established that Rho ATPase activity has a V_max of 30 s⁻¹ at 22 °C (9). Rho has a single class of three ATP binding sites that are catalytic sites (10, 11) with a K_d of 1 μM and based on crystal structure (5), three more sites that may have ATP only at millimolar levels. During steady-state V_max ATP hydrolysis, three molecules of ATP are bound per hexamer (12).

To determine which one or more aspects of the protein’s action limits the speed of catalysis, we measured ATP binding and the rates of ADP and P_i release from Rho. We found that, at physiological ATP concentration, the ATP on-rate and P_i hydrolysis product dissociation are not rate-limiting; they are faster than k_cat. The ADP off-rate, however, is of the same magnitude as catalysis and could be a determinant of V_max.

EXPERIMENTAL PROCEDURES

Wild-type Rho from E. coli was prepared as previously described (13) from strain AR120/A6 containing plasmid p39ASE (14). The concentration of Rho was spectrophotometrically determined using ε_max = 3.25 cm⁻¹ at 280 nm. The preparations used had specific activities with poly(C) at 37°C of 10–20 units mg⁻¹. A unit of activity is the amount of enzyme that hydrolyzes 1 μmol of ATP in 1 min.

2',3'-O-[(N'-Methylanthraniloyl)-ATP] (mantATP) and mantADP were from Jena Bioscience, Germany. In our standard V_max Rho ATPase assay in the absence of ATP, mantATP was transformed into a faster-migrating compound on TLC (see below) plus P_i, consistent with it serving similarly to ATP as substrate for the hydrolysis activity of Rho. It has thus been used as a Rho substrate (15). [γ-32P]ATP and [γ-32P]mantATP were synthesized by an exchange reaction and subsequently purified (Ref. 17, as modified previously (18)). Specific activities ranged from ~10⁵ to 10⁶ cpm/nmol. The mantATP exchange reaction was allowed to proceed at room temperature for 6 h, followed by purification through a Sephadex LH20 col-
um (Amersham Biosciences) in water (similar to purification of 2′,3′-O-(2,4,6-trinitrophenyl)-ATP in Ref. 18). The final fractions were analyzed by TLC (Polygram CEL 300 PEI/UV254, Machery-Nagel, Germany; in 2 m formic acid/0.5 m LiCl and quantified using a Packard Cyclone phosphorimaging device (PerkinElmer Life Sciences)). The final combined fractions were found to have <3% of radioactivity in P_i.

RNA was poly(C) from Amersham Biosciences, dissolved in water and used without further purification. Its average size was 400 bases as stated by the manufacturer. It was typically used in 2- to 30-fold excess of 100-base long equivalents over Rho hexamer. The buffer used was TAGME (40 mM Tris acetate, pH 8.2 at 37 °C, 150 mM potassium glutamate, 1 mM magnesium acetate, 0.1 mM EDTA).

**ATP Hydrolysis by Rho**—Rho ATPase activity was measured using [γ-32P]ATP and monitoring the production of 32P_i (10). Reaction samples were typically quenched by addition of trichloroacetic acid to a final concentration of 2.5% (w/w). 32Pi product was separated from [γ-32P]ATP by mixing with acid-washed charcoal in water at a final concentration of 2.5% (w/v) and sampling the supernatant after centrifugation at 14,000 × g for 2 min.

**On-rate of ATP**—A rapid mix/chemical quench apparatus (model 1500/1501/1502, Update Instrument, Madison, WI) (19, 20) with either a Wiskind or T mixer was used at room temperature (21–23 °C, Scheme 1). Rho was mixed with [γ-32P]ATP, and, after various times, excess unlabeled ATP and RNA were added to promote hydrolysis of bound [γ-32P]ATP. Trichloroacetic acid and charcoal were then used as in the other experiments to measure 32P_i production.

Equal volumes of Rho (1.9 mg/ml and 20 μM active sites) in one syringe of the rapid mix instrument and [γ-32P]ATP (20 μM or 40 μM, 1 × 10^6 cpm/nmol) in the other syringe were mixed and passed through an aging hose into 240 μl of rapidly stirred trap solution, which was 18 mM unlabeled ATP, 20 mM magnesium acetate, 0.15 mg/ml poly(C) in TAGME buffer. 2 s later, 160 μl of 50% trichloroacetic acid was added to quench the reaction. From each 500-μl quenched mixture, 50 μl was counted for total radioactivity, and 50 μl was treated with 400 μl of 8% w/w charcoal to determine the amount of 32P_i produced. In control experiments to measure background, [γ-32P]ATP (20 μM or 40 μM) in TAGME buffer was injected into trap solution. Data were analyzed according to Equation 1 (21) for a second order reaction,

\[
[1/(B_0 - A_0)] \ln[A_0(B_0 - x)/B_0(A_0 - x)] = kt
\]

where \(A_0\) and \(B_0\) are the starting concentrations of molecule A and molecule B, \(x\) is the concentration of AB complex at time \(t\), and \(k\) is the observed rate. \(k\) was obtained from the slope of the plot of \(\ln(\text{ATP}_{\text{free}}/\text{Rho}_{\text{free}})\) versus time.

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**Scheme 1**

**Scheme 2**

**E. coli Rho Mechanism**

**Off-rate of ATP from Rho**—In a stopped-flow spectrofluorometer (Model SF-2003, KinTek Corp., Austin, TX), a mixture of Rho (0.5 μM in hexamer) plus mantADP (1.5 μM) plus poly(C) (0.05 mg/ml) in TAGME buffer, in one syringe of the apparatus, was mixed at room temperature with an equal volume of 1 mM ATP or 1 mM ADP in the same buffer plus 1 mM magnesium acetate, in the other syringe (Scheme 3). The fluorescence emission at 450 nm was monitored using a 400 nm cutoff long pass filter (Model 57345, Spectra-Physics Oriel Corp., Stratford, CT) with excitation at 360 nm. The experiments were also done with poly(C) placed in the syringe with ATP or ADP instead of Rho and mantADP. Release of mantADP from Rho in the absence of RNA was also studied. In control experiments to measure fluorescence background, Rho was in one syringe, poly(C) and ATP in the other syringe, and no mantADP was used. The mantADP dissociation kinetic data were fit to a single exponential function (Equation 3) or to the sum of two exponential functions (Equation 4).
**mattATP Hydrolysis by Rho**—[γ-32P]mattATP hydrolysis by Rho was measured as in the V∞ max assay for poly(C)-dependent ATP hydrolysis by Rho (10) by quantitation of the 32Pi produced at room temperature in the presence of poly(C) as the required RNA cofactor. Each 50-μl assay mixture in TAGME buffer was 1.1 mM [γ-32P]mattATP (~6000 cpm/nmol), 1 mM magnesium acetate, 2 μg/ml in poly(C), and contained 25 ng of Rho. All ingredients except Rho were mixed and preincubated at room temperature for 5 min. After 15 min of incubation with Rho, the reaction was quenched by the addition of 250 μl of ice-cold 5% (w/w) trichloroacetic acid and 150 μl of 8% charcoal. Following centrifugation, a sample of the supernatant containing 32Pi was counted in a liquid scintillation counter. The V∞ max assay with [γ-32P]ATP was performed as a control. Background 32Pi was obtained by replacing protein solution with TAGME buffer in the assay.

**mattATP Binding to Rho**—mattATP equilibrium binding measurements were conducted at room temperature using Microcon-30 ultrafiltration apparatus (Millipore) (see Ref. 11). Each 100-μl binding mixture was 1 μM Rho (in subunits) and 0.25–12 μM [γ-32P]mattATP with specific activity of 1.8 × 105 cpm/nmol. After the binding mixture was incubated at room temperature for 1 min, ~70 μl was applied on the Microcon membrane, which was then centrifuged to separate bound from free [γ-32P]mattATP. The data were analyzed by Scatchard analysis (bound/free was plotted versus bound/Rho subunit).

\[ P_i - \text{Off-rate} \]—The Pi off-rate (a–d) was measured using a fluorescently tagged form of the A197C mutant of *E. coli* phospho-binding protein (PBP) and protocols similar to those previously published (23–25). We obtained the strain ANCC75 with the plasmid pSN5182 encoding the mutant PBP from Martin Webb, MRC, Mill Hill, UK; this strain was induced to overproduce PBP by phosphate limitation as described (24) and was labeled with the coumarin N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC), available from Molecular Probes, Inc. (Invitrogen) as described before (25) as follows. (a) Expression and purification of PBP: ANCC75 was grown as described (24); in high phosphate TG plus medium, the culture required ~12 h at 37°C to achieve an A600 of 1.5. Following PBP induction in low phosphate medium, PBP was released from cells by osmotic shock (24) and purified as described (26). The final PBP solution was stored as 50- to 100-μl aliquots at ~70°C. PBP concentrations were determined by measuring absorbance at 280 nm using an extinction coefficient of 17.8 (mg/ml) cm−1 (24). ~40 mg of PBP was purified from 13 g of wet cell pellet. (b) Synthesis and purification of MDCC-PBP: PBP was labeled with MDCC, and MDCC-PBP was purified from the labeling reaction mixture (25). The final product was stored as 50- to 100-μl aliquots at ~70°C. ~32 mg of pure MDCC-PBP (based on a single band on SDS-PAGE gel) was obtained from 35 mg of PBP. (c) Pi mop to reduce P, contamination: To measure P, release from Rho using MDCC-PBP, adventitious P, must be meticulously removed from the reagents and also the surfaces of instruments. Because poly(C) is involved in the reaction, all precautions to minimize RNase activity are also taken (diethyl pyrocarbonate treatment to inactive ribonucleases). P, mop composed of 7-methylguanosine (1 mM) and PNP (1 unit/ml) was used to remove P, (23, 25) by conversion to ribose 1-phosphate, which binds poorly to PBP. Concentrated MDCC-PBP and Rho in separate dialysis sacs (Spectra/Per) were dialyzed against 40 volumes of TAGME containing P, mop at 4°C for 24 h. P, was removed from the larger volumes of TAGME buffer and from concentrated ATP solutions by placing P, mop (~1/5 volume) inside a dialysis bag. The treated enzymes and reagents were assayed for P, using a P,Per phosphate assay kit, sensitive down to 0.2 μM P, (Molecular Probes), following the manufacturer’s protocol. The fluorescence produced was detected by a Wallac 1420 VICTOR3 fluorescence microtiter plate reader (PerkinElmer Life Sciences) with excitation at 530 nm (using 530 nm excitation filter for VICTORTM, catalogue no. 1420-502) and emission at 590 nm (with 590 nm emission filter for VICTORTM, catalogue no. 1420-544). The stopped-flow syringes and sample lines were treated with P, mop for 30 min, and then rinsed with ~10 ml P, mop-treated TAGME buffer. (d) Stopped-flow kinetics of P, dissociation from Rho (Scheme 4): Phosphate release kinetics were measured in the KinTek stopped-flow spectrofluorometer using a fluorescence-coupled assay (23, 25). Rho was rapidly mixed with ATP and RNA in the presence of MDCC-PBP. Following hydrolysis of the ATP by Rho, when P, is released from the active site, MDCC-PBP traps the free P, and exhibits a fluorescence increase.

A standard curve for P, MDCC-PBP fluorescence was determined by stopped-flow experiments in which 20 μl of a premix of 9 μM MDCC-PBP (concentration in the syringe) plus 0.04 unit/ml PNP and 0.2 mM 7-methylguanosine in TAGME buffer was mixed with 20 μl of various concentrations of P, solution (0.5–11 μM) in TAGME buffer at room temperature. The excitation wavelength was 425 nm, and the fluorescence emission at 464 nm was detected using a 450 nm cutoff long pass filter (Model 57347, Spectra-Physics Oriel Corp.).

To find the off-rate of P, from Rho, in one syringe of the stopped-flow instrument was 1.5 μM Rho hexamer, 9 μM MDCC-PBP, 0.02 unit/ml PNP, and 0.1 mM 7-methylguanosine in TAGME buffer; the other syringe held 200 μM ATP, 0.15
mg/ml poly(C), 0.02 unit/ml PNP, and 0.1 mm 7-methylguanosine in TAGME buffer. The measurements were also made with poly(C) placed in the syringe with Rho and MDCC-PBP instead of in the syringe with ATP. 5–7 scans were averaged to obtain the final traces. The beginning part of the trace (0–0.03 s) was fit to a single exponential function to determine the rate of Pi release. The control reactions to determine adventitious background included 1) Rho and MDCC-PBP in one syringe and poly(C) in the other syringe and 2) Rho and MDCC-PBP in one syringe and ADP and poly(C) in the other syringe.

**KINSIM (27) Modeling of Pi Dissociation Rates**—Data for Pi binding to MDCC-PBP were compared with the curves produced using a model in which Pi binding was preceded by a step to trap ATP. Several KINSIM simulations were carried out as diagrammed in Scheme 1. Rapid mix/chemical quench experiments (Scheme 3) were used to measure total radioactivity; the remaining solution was applied to a 1-ml disposable syringes that had been filled with Sephadex G-50 and centrifuged at 100 × g for 2 min (28). The syringe was then centrifuged identically a second time to separate unbound small molecules (which remain in the column) from those bound to Rho (which elutes from the column). The radioactivity in the filtrate was determined in a liquid scintillation counter.

**RESULTS**

**ATP On-rate for Rho**—Rapid mix/chemical quench experiments were carried out as diagrammed in Scheme 1. Rapid mixing of 10 or 20 μM [γ-32P]ATP with 3.3 μM Rho hexamer was followed by aging for 50–200 ms. The mixture was then injected into a stirred trap containing RNA plus 10 mm nonradioactive MgATP. Under these conditions, any [γ-32P]ATP bound to Rho is hydrolyzed, whereas non-bound radioactive ATP is diluted 1000-fold by trap ATP. After 2 s, trichloroacetic acid is added to quench the reaction, and product 32Pi is determined as a measure of the amount of radioactive ATP that bound to Rho during the aging step. To obtain the on-rate constant, the data for each concentration of [γ-32P]ATP were analyzed for a second-order reaction according to Jencks (21). Data are shown in Fig. 1; the rates are from the slopes of least-squares-fit lines. The results from the two concentrations of ATP agree and indicate that the on-rate constant is 2.3–2.4 × 107 M⁻¹ s⁻¹.

**ATP Off-rate from Rho ATP Complexes**—Using the above on-rate constant and the previously measured Kd for ATP under similar conditions, 1 μM (9), an ATP Koff of 0.24 s⁻¹ is calculated from Kd = Koff/KA, which corresponds to a half-life of 2.9 s. The following experiment was performed to check the magnitude of the off-rate.

In manual rapid mix/chemical quench experiments (Scheme 2), Rho[γ-32P]ATP₃ complexes were mixed with non-radioactive ATP and chased for 2 or 5 s or 2 min, RNA was added to permit hydrolysis of Rho-bound ATP (trap), and the reaction was finally quenched with trichloroacetic acid. We note that the value obtained from this experimental design could be a composite of the ATP dissociation rates from both Rho-ATP and Rho-ATP-RNA complexes. Correction for rebinding of [γ-32P]ATP and its hydrolysis during the trap was made by quenching with trichloroacetic acid at 5, 10, and 20 s after RNA addition and extrapolating the linear data back to t = 0 (data not shown). No remaining bound [γ-32P]ATP was found when this extrapolation was made with data from 5-s and 2-min chase times (data not shown). The 2-s chase time was the fastest we could reliably achieve in a manual experiment, yielding a lower limit for the ATP off-rate of 0.8 ± 0.05 s⁻¹ (t½ = 0.8 s), in fair agreement with the 0.24–2.9 s values calculated above.

**ADP Off-rate**—ADP analogue mantADP was employed in stopped-flow fluorescence experiments (Scheme 3) to measure ADP release from the Rho·ADP intermediate (or from the Rho-RNA·ADP intermediate when RNA was present). mantADP fluorescence is quenched by the buffer; therefore, when Rho plus mantADP was mixed with a large excess of ATP in a stopped-flow machine, the decrease of fluorescence indicates mantADP release from Rho active sites (29, 16). Variations of this experiment involved inclusion of RNA in the mantADP-
Rho mixture, use of ADP instead of ATP in the second solution, and experiments in the absence of RNA (non-hydrolysis conditions).

The results are presented in Fig. 2 and Table 1. The fluorescence traces fit well to a single exponential function (Equation 3) and to the sum of two exponentials (Equation 4). In the absence of RNA, information from fluorescence amplitudes ($A_1$ and $A_2$ in Table 1) indicates that $k_2$ contributes the major part to the overall rate, and a single exponential fit is indicated. For experiments that included poly(C), $A_1$ was close to $A_2$, indicating a significant contribution from two events. Although it would be expected that a function with a single exponential would best fit the data, a fit by the sum of two exponentials is not ruled out because mantADP is a 70:30 mixture of two isomers, 3'-mant-ADP and 2'-mant-ADP (30), which could interact differently with Rho (16). The currently available structures for Rho do not clarify this possibility: nucleotides bind at Rho subunit interfaces (5) in a very open protein configuration; modeling using Swiss-Pdb Viewer shows that there is sufficient space for either 2'- or 3'-mantADP (not shown). What happens when the protein closes around its substrate to perform hydrolysis is not known. A possible alternative explanation, that there is more than one type of mantATP binding site on Rho, was ruled out by [$\gamma$-$^{32}$P]mantATP binding experiments, whose results were similar to those obtained with ATP: 3 equivalent mantATP binding sites on Rho with $K_D \sim 1.5$ mM (data not shown).

We measured the $V_{\text{max}}$ of mantATP hydrolysis by Rho at 21 °C as 4.3 s$^{-1}$, compared with 30 s$^{-1}$ for ATP (data not shown). Presumably this decrease is because the additional ring structure on the ribose of mantATP affects its interaction with Rho, perhaps slowing its off-rate. The measurements of mantADP release from Rho in the presence of poly(C), 15–20 s$^{-1}$ (Table 1, fitting a single exponential function), are all at least 3-fold faster than this steady-state mantATP hydrolysis rate. This is not a large factor, so mantADP release may make a significant contribution to rate limitation, although it is not necessarily the sole rate-limiting step in the mantATP hydrolysis pathway.

$P$, Binding—In previous work using RhoE155K, we found the $K_D$ for $P$, to be 30–40 mM (10) by measuring its ability to displace ATP from Rho and to competitively inhibit its ATPase reaction. Here we checked this measurement using wild-type Rho and a centrifuge column (28) to separate free and Rho-bound $^{32}$P$\gamma$. Using 10 μM Rho subunits and 30 mM $P_\gamma$, we found...
E. coli Rho Mechanism

**TABLE 1**

| Experiment                  | Single exponential fit | Double exponential fit |
|-----------------------------|------------------------|------------------------|
|                             | $k_1$ | $k_2$ | $A_1$ | $k_3$ | $A_2$ |
| (Rho + mantADP) + (polyC + ATP) | 20.8   | 42.7  | 0.6   | 12.2  | 0.4  |
| (Rho + mantADP) + (polyC + ATP) | 17.1   | 71.7  | 0.6   | 8.2   | 0.4  |
| (Rho + mantADP) + (polyC + ATP) | 14.7   | 30.9  | 0.4   | 10.3  | 0.6  |
| (Rho + mantADP) + (polyC + ATP) | 19.5   | 34.1  | 0.8   | 4.9   | 0.2  |
| (Rho + mantADP) + (ADP)      | 1.3    | 18.9  | 0.1   | 1.3   | 0.9  |
| (Rho + mantADP) + (ADP)      | 1.2    | 15.8  | 0.1   | 1.2   | 0.9  |

Table 1: manitADP release rate from Rho

Fluorescence was measured over time after mixing the solutions described under “Experimental Procedures” using a stopped-flow device. Parentheses indicate that the components enclosed were premixed in the syringe. The data (Fig. 2) were fit to single- and double-exponential models (Equations 3 and 4), and the parameters obtained from the best fits are presented.

negligible counts associated with Rho, regardless of whether ADP and poly(C) were present. This result suggests that either the $P_1$ $K_{DP}$ for wild-type Rho is at least 5-fold higher than 30 mM, or the $P_1$ off-rate is faster than 0.2 s$^{-1}$ (10) (or both).

$P_1$ Off-rate $= 0.75$ $\mu M$ Rho hexamer plus 4.5 $\mu M$ of the fluorescent $P_1$ indicator MDCC-PBP were mixed with 100 $\mu M$ ATP plus RNA, conditions in which a hydrolysis burst of 1 molecule of ATP per Rho hexamer was expected, followed by steady-state ATP hydrolysis (Scheme 4). The appearance of fluorescence was monitored; typical results are shown in Fig. 3 (A and B). As in the mantADP off-rate experiments, different combinations and orders of mixing of reagents were employed. For both experimental samples and $P_1$ alone, a fast fluorescence increase occurred within 10 ms after starting the assay. In experimental samples, this was followed by a slower signal increase and then a plateau. The fast fluorescence increase represents the binding of product $P_1$ that was released from Rho during a burst of pre-steady-state ATP hydrolysis, to MDCC-PBP. During the slower signal increase phase, Rho is performing steady-state ATP hydrolysis and $P_1$ released from Rho is trapped by MDCC-PBP. Because all the MDCC-PBP in the assay associates with $P_1$, the fluorescent signal plateaus. The initial phase of fast fluorescent increase was fit to a single exponential function (Equation 3) from which the rate of $P_1$ release was obtained: $269.7 \pm 4.2$ s$^{-1}$ and $236.5 \pm 3.9$ s$^{-1}$ for the two experimental designs used. As a control to show that the presence of MDCC-PBP does not affect Rho ATPase activity, a $V_{\text{max}}$ assay for poly(C)-dependent ATP hydrolysis by Rho was done in the presence of MDCC-PBP, similar to stopped-flow conditions. No effect was found (data not shown).

The association rate constant for $P_1$ and MDCC-PBP obtained from experiments with $P_1$ alone was $6 \times 10^7$ M$^{-1}$s$^{-1}$ (21$^\circ$C, TAGME buffer; 2.5–11 $\mu M$ $P_1$ concentrations were used in separate experiments, and the association rate constant was obtained from the slope of a plot of rate of initial fluorescence increase versus $P_1$ concentration; data not shown), which is in agreement with $1.36 \times 10^8$ M$^{-1}$s$^{-1}$ (21$^\circ$C, 10 mM PIPES buffer, pH 7.0) previously published (23). The rate in Fig. 3E, at 3 $\mu M$ $P_1$, is $250$ s$^{-1}$. In these experiments, fluorescence depends on several steps, including ATP hydrolysis by Rho, $P_1$ release from Rho, and $P_1$ binding to MDCC-PBP. The kinetics in the $P_1$ standard curves and in the experimental samples indicates that the $P_1$ off-rate from Rho is faster than could be measured by this technique and is far faster than the $30$ s$^{-1}$ $k_{cat}$.

**DISCUSSION**

The present work measures rate constants for several steps during RNA-dependent ATP hydrolysis by Rho. Scheme 5 shows the most likely in vivo pathway. The first two steps constitute ligand binding to form the Rho-ATP-RNA complex. In step 3, bound ATP is hydrolyzed to ADP and $P_1$. Steps 4–6 are hydrolysis products and RNA release steps. After step 6, a possible protein conformational change is indicated that precedes the next hydrolysis cycle.

$k_1$ is the equilibrium constant for ATP binding to Rho in the absence of RNA. At millimolar in vivo ATP concentration (31), ATP binding is not the rate-limiting step: $2.4 \times 10^8$ M$^{-1}$s$^{-1}$ $\times 10^{-3}$ M yields an on-rate of 240 s$^{-1}$.

In step 2, RNA binds to Rho already filled with ATP. There are two classes of RNA binding sites on Rho: “primary” and “secondary” (32–37). RNA binding at the secondary site is thought to be more directly involved in ATP hydrolysis (32, 38) and is what is represented in Scheme 5. Normally it is a further interaction by an RNA molecule that is already bound in the primary site. We do not have a direct measurement of the rate of this step, but two observations suggest that it is fast: 1) Based on the presence of a burst of ATP hydrolysis upon mixing Rho-ATP$P_1$ complexes with RNA (9, 12, 16) and the necessity of RNA binding in secondary sites for ATP hydrolysis (32), RNA binding must occur faster than the chemistry step of the ATPase reaction, which is at least 300 s$^{-1}$ (9, 2). In the experiments of Fig. 3B, which measure the $P_1$ off-rate, RNA binding and ATP hydrolysis are included in the $P_1$ release measurement, so they must occur at least as fast or faster than the dissociation of product $P_1$. (As discussed below, the $P_1$ off-rate is likely of the order of 1000 s$^{-1}$). Kim and Patel (39) observed intrinsic protein fluorescence changes upon addition of RNA to Rho that were interpreted as four different configurations of Rho-RNA complexes. The first fluorescence change is associated with RNA binding in Rho primary sites, but it is not clear which of the other fluorescence changes might be relevant to secondary site binding. Thus we are not able to use the rates they found.

The equilibrium constant of the catalytic step, $K_y$, was estimated to be 1 (10), and the burst indicates that one or more steps after chemistry are rate-limiting. Hydrolysis ($k_3$) must occur at least at 300 s$^{-1}$ to be consistent with the burst kinetics (9). Also, because ATP hydrolysis is included in the $P_1$ off-rate measurement of Fig. 3B, the hydrolysis step must be faster than $P_1$ release.

Steps 4–6 are product and ligand release steps after catalysis. The order in which they occur is not known. The $P_1$ release results require that the $P_1$ off-rate ($k_4$) is at least as fast as $P_1$ binding to PBP, $\sim 250$ s$^{-1}$ (Fig. 3). KINSIM simulations suggest that the off-rate is at least 3–4 times the rate of $P_1$ binding to PBP, otherwise the initial part of the $P_1$ detection curve would be measurably slowed (not shown). Thus the $P_1$ off-rate is of the order of 1000 s$^{-1}$ or faster. Adelman et al. (40) obtained distinctly different results for the same experiment: they found a 20- to 30-ms lag in $P_1$ release. They do not provide a $P_1$ binding
to PBP control for comparison, but their observed experimental lag is twice the length of the lag in our control (Fig. 3E). The explanation for these different results is not known.

ADP dissociates from Rho in step 5; the off-rate for ADP ($k_5$) must be at least as fast as the overall steady-state hydrolysis rate ($30\,s^{-1}$ at $22^\circ C$). The off-rate for mantADP is about three times the mantATP hydrolysis rate; if a similar relationship exists between ADP release and ATP hydrolysis, then ADP release would be $90\,s^{-1}$. Another estimate of the ADP off-rate is obtained from analysis of steady-state catalysis data. We found an average of three molecules of ATP bound to Rho hexamers during steady-state catalysis (12, 41). KINSIM modeling shows that the ADP off-rate affects this steady-state value: an off-rate slower than $\sim 100\,s^{-1}$ results in $<75\%$ saturation of Rho hexamers with ATP, a level that would have been discernible from the observed $100\%$ (12). This $100\,s^{-1}$ lower limit is consistent with the $90\,s^{-1}$ rate predicted if the ADP off-rate is three times the ATP hydrolysis rate.

In step 6, RNA is released from the Rho secondary site, and $k_6$ should also be at least as fast as the catalytic cycle, $30\,s^{-1}$. Using this value with the measured $C_{10}$ oligomer $K_D$ for RNA secondary sites ($7\,\mu M$ (37)), $k_{on}$ is calculated as $\sim 4\times 10^6\,M^{-1}s^{-1}$. However, the $C_{10}$ $K_D$ was determined with only Rho and $C_{10}$ present, whereas in the current work, the primary Rho binding site was filled with long RNA polymer and the secondary sites presumably by other portions of the same RNA molecule. The enzyme form may not be the same under these two conditions, so it is not clear whether the oligomer binding values are applicable.

We have included a protein conformation change as a discrete step in Scheme 5 (step 7). We have no data concerning the existence or placement of such a step in the overall scheme. Protein conformation changes could and very well may occur at any and at more than one step. Our present data support a conformation change that is rate-limiting only following $P_i$ release, for example, in concert with RNA release.

All of the forward rate constants reported here (Table 2) are faster than the rate of ATP hydrolysis by Rho, $30\,s^{-1}$, although the $90\,s^{-1}$ ADP off-rate may contribute to the rate limitation. As yet unmeasured RNA dissociation and a hypothetical protein conformation change are also candidates for a rate-deter-


E. coli Rho Mechanism

TABLE 2
Values of kinetic constants for steps during ATP hydrolysis by Rho (Scheme 5)

| Step                              | $K_1$      | $k_{f1}$     | $K_{on}$ |
|-----------------------------------|------------|--------------|----------|
| 1. ATP association                | $1 \times 10^6$ M | $2.4 \times 10^5$ M s$^{-1}$ | 0.24–0.8 s$^{-1}$ |
| 2. RNA 2° site association        | ~1         | ~$10^5$ s$^{-1}$ | 10 s$^{-1}$ |
| 3. Chemistry                      | ~3 x $10^{-2}$ M | ~$10^5$ s$^{-1}$ | 3 x $10^4$ s$^{-1}$ |
| 4. P$_i$ release                  | 30 x $10^{-6}$ M | ~$10^5$ s$^{-1}$ | 3 x $10^4$ s$^{-1}$ |
| 5. RNA release                    | >30 s$^{-1}$ |               |          |
| 6. Protein conformation change    | >30 s$^{-1}$ |               |          |

mining step. Excluded from this analysis is RNA binding in the primary site of Rho, although binding to and release from this site could be important to the movement of Rho along RNA. Binding is fast (39), but release is also a candidate for a rate-limiting step.

Of interest is the finding that the mantADP release rate is 16-fold faster in the presence of poly(C) than with Rho alone (Table 1, comparing $K_1$ values from the single exponential fits). This result suggests that a conformational change to open the nucleotide binding site for product release is faster when RNA is bound, which in turn suggests that the hexamer is structurally different when it is in a complex with RNA.

There is a possible concern that the pre-steady-state rates measured here could be different from those during steady-state catalysis. In particular, because Rho displays catalytic cooperativity (9), where events in one active site affect the rate of events in other active sites, one could speculate that the first ATP hydrolysis is different from subsequent catalytic cycles, because it is not preceded by catalysis in another active site. We have demonstrated, however, that a preceding catalysis is not required for $V_{max}$ ATPase activity by Rho. The key to catalytic cooperativity in Rho is the simultaneous binding of an ATP-like molecule in all three active sites. Once this has occurred, a protein conformation is apparently achieved that permits sequential $V_{max}$ ATP hydrolysis in the three active sites (12). It thus seems likely that the rates during the first enzyme turnover are the same as those during the $V_{max}$ steady-state.

In summary, measurement of substrate and product on- and off-rates indicates that ADP dissociation, perhaps together with either the RNA off-rate or a protein conformation change, are rate-limiting for RNA-dependent ATP hydrolysis by Rho.

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