Catalytic Cooperativity among Subunits of *Escherichia coli* Transcription Termination Factor Rho

**KINETICS AND SUBSTRATE STRUCTURAL REQUIREMENTS**

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*Escherichia coli* transcription termination factor Rho shows a 30-fold faster rate of ATP hydrolysis when all three catalytic sites are filled with ATP than when only a single site is filled (Stitt, B. L. and Xu, Y. (1998) *J. Biol. Chem.* 273, 26477–26486). To study the structural requirements of the substrate for this catalytic cooperativity, rapid mix/chemical quench experiments using various ATP analogs were performed. The results indicate that it is the configuration of the β- and γ-phosphoryl groups of ATP that is of primary importance for the rate enhancement. Our results also show that there are kinetically slow branches of the enzyme mechanism that are not seen when the chemistry step of the catalytic cycle is fast. These branches become prominent, however, when two of the three Rho active sites are empty or bear non-hydrolyzable compounds. A first-order step that is slow compared with $V_{\text{max}}$ catalysis enables a single ATP molecule bound in any one of the three Rho active sites to be hydrolyzed and defines the kinetically slow branches. This first-order step could be a protein conformation change or a rearrangement of bound RNA. The results reinforce the importance of catalytic cooperativity in normal Rho function and suggest that several protein conformational changes exist along the catalytic pathway.

Catalytic cooperativity occurs in multisubunit enzymes when a catalytic step in one active site affects the catalytic cycle in another active site. It is best documented in F$_{1}$-type ATPases of mitochondria. When ATP is bound in only one of the three active sites, it is reversibly hydrolyzed by the enzyme without release of products until ATP binds in at least one additional catalytic site. The binding of the additional ATP molecule(s) increases the rate of product release from the first active site, completing the catalytic cycle there (1, 2).

Catalytic cooperativity has been reported for *Escherichia coli* transcription termination factor Rho (3). Rho aids in the release of newly synthesized RNA from paused transcription complexes (reviewed in Ref. 4). The homohexameric Rho protein binds nascent RNA and, with the RNA-dependent hydrolysis of ATP, travels 5′ → 3′ along the RNA to disrupt the ternary transcription complex, releasing product RNA and allowing RNA polymerase to recycle. RNA-dependent ATP hydrolysis by Rho can be studied in a simplified system, requiring only Rho, RNA, and MgATP. During the course of pre-steady-state rapid mix/chemical quench experiments carried out with such a simplified system, Stitt and Xu (3) found that Rho exhibits catalytic cooperativity. The steady-state rate of RNA-dependent ATP hydrolysis is 30-fold slower when a single ATP molecule is bound to the Rho hexamer than when all of the ATPase sites are filled.

Here, the molecular features that are required of a nucleotide for it to enhance or “promote” the hydrolysis rate of a single Rho-bound ATP molecule are explored. Results from experiments using various ATP analogs and related compounds are considered in relationship to the present model for ATP hydrolysis by Rho.

**EXPERIMENTAL PROCEDURES**

Enzymes, Buffers, and Substrates—Wild type Rho from *E. coli* was purified as described previously (5) from strain AR120/A6 containing plasmid p90ASE (6). The concentration of Rho was spectrophotometrically determined using $A_{280}$ nm = 3.35 cm$^{-1}$ (7). The enzyme preparations used had specific activities with poly(C) at 37 °C of 10–20 units mg$^{-1}$. A unit of activity is defined as that amount of enzyme that hydrolyzes 1 μmol of ATP in 1 min.

Buffers were TKME (40 mM Tris-HCl, pH 7.7 at 25 °C, 50 mM KCl, 1 mM MgCl$_2$, 0.1 mM EDTA) and TAGME (40 mM Tris acetate, pH 8.3 at 25 °C, 150 mM potassium glutamate, 1 mM Mg acetate, 0.1 mM EDTA). Buffers were supplemented with MgCl$_2$ or Mg acetate at a concentration equimolar with that of any added nucleotide or phosphate ester.

Poly(C) RNA with an average length of 400 bases was from Amersham Biosciences and was dissolved in water at 5 mg/ml. [γ-$^{32}$P]ATP at a specific activity of 1–10 Ci mmol$^{-1}$ was synthesized from [γ-$^{32}$P] and ATP according to the exchange method of Glynn and Chappell (8) as modified by Grubmeyer and Penefsky (9). Following purification, these preparations typically had <3% of their radioactivity in compounds other than ATP. [γ-$^{32}$P]CTP was prepared similarly, although the reaction was slow (~3 h rather than 30 min) and only ~50% exchange was achieved rather than >90%.

[γ-$^{32}$P]Sodium pyrophosphate (PP),† was NEX019 from PerkinElmer Life Sciences and was purified by ion-exchange chromatography to remove PP$^\circ$, PP$^\circ$, ACS grade, was from Fisher Scientific.

ADP (disodium salt, Roche Applied Science) was found to contain sufficient ATP to affect results. The contaminating ATP was decreased by conversion to ADP + glucose 6-phosphate as follows. To 1.5 ml of 85 mM ADP adjusted to pH 7.5 with Tris base was added equimolar Mg acetate, glucose to 11 mM, and 5 units of hexokinase (H-4502, Sigma). Following incubation at 30 °C for 5 h, hexokinase was removed by filtration using Amicon Centricon-30 concentrators at 6000 rpm in an

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SS34 rotor for 20 min. The removal of hexokinase was confirmed by the stability of a trace amount of \( [\gamma-32P]ATP \) that was added to a portion of the filtrate.

The buffer from Sigma (A1388) was also treated with hexokinase. To avoid consumption of the ATP-S\(_5\) during this treatment, the incubation period with hexokinase was minimized as determined by trials in which the hydrolysis of \( [\gamma-32P]ATP \) added at 8 \( \mu \)M final concentration to a sample of the ATP-S\(_5\) stock solution was monitored. Depletion of the labeled ATP was measured (10) by quenching reactions with trichloroacetic acid, adding charcoal to precipitate adenine nucleotides, centrifuging, and measuring radioactivity in \( [\gamma-32P] \) glucose-6-phosphate in the supernatant by liquid scintillation spectrometry. The treatment thus determined for 1.8 ml of a 14 mM solution of ATP-S\(_5\) in 10 mM Tris-HCl, pH 8.5, 5 mM dithiothreitol, 14 mM Mg acetate, 14 mM Mg chloride was incubation for 20 min at 32 °C with 50 units of hexokinase followed by ultrafiltration as described above to remove the enzyme. Following thin-layer chromatography, the resulting ATP-S\(_5\) appeared visually to contain 10% ATP.

For AMP-PNP (A-2647 from Sigma), 2.4 ml of 33 mM AMP-PNP in 10 mM Tris-HCl, pH 8.0, 42 mM Mg acetate, 12.5 mM Mg chloride was treated for 16 h at 25 °C with 10 units of hexokinase. For ddATP (lithium salt, D5413, Sigma), 130 \( \mu \)M of an 8 \( \mu \)M solution in TAGME buffer with additional Mg acetate to 12 mM and glucose to 15 mM was treated with 2.5 mg of hexokinase at 30 °C for 20 min.

CTP, (triptosium salt) was from Calbiochem. The disodium salt from Sigma was also used. UTP was from P-L Biochemicals. GTP, CDP, 2'-deoxyadenosine 5'-triphosphate (2'-dATP, D6500), 3'-deoxyadenosine 5'-triphosphate (3'-dATP, corydine 5'-triphosphate, C9137), adenosine 5'-(\( \beta, \gamma \))-methylene triphosphate (ATPP, AMP-PCP, M7510), adenosine 5'-(\( \alpha, \beta \))-methylene triphosphate (AMP-CPP, lithium salt, M6517), idodiphosphate (PNP, I0631), and triphosphate (PPP, 98%, T5633) were obtained from Sigma. Adenosine tetraphosphate (ATPP) was from Jeni Bioscience (Jena, Germany). Adenine arabinoside-5'-triphosphate was the kind gift of R. J. Suhadolnik (Temple University).

Rapid mix/chemical quench experiments (11) were carried out at 23 °C as described (3). Typically, one syringe of the Update Instruments T5633) were from Sigma. Adenosine tetraphosphate (ATPP) was from BioMol. The disodium salt from Sigma was also used. UTP was from P-L Biochemicals. GTP, CDP, 2'-deoxyadenosine 5'-triphosphate (2'-dATP, D6500), 3'-deoxyadenosine 5'-triphosphate (3'-dATP, corydine 5'-triphosphate, C9137), adenosine 5'-(\( \beta, \gamma \))-methylene triphosphate (ATPP, AMP-PCP, M7510), adenosine 5'-(\( \alpha, \beta \))-methylene triphosphate (AMP-CPP, lithium salt, M6517), idodiphosphate (PNP, I0631), and triphosphate (PPP, 98%, T5633) were obtained from Sigma. Adenosine tetraphosphate (ATPP) was from Jeni Bioscience (Jena, Germany). Adenine arabinoside-5'-triphosphate was the kind gift of R. J. Suhadolnik (Temple University).

The basic experiment to determine the features of the ATP molecule that are needed for promotion employed rapid mix/chemical quench techniques. Rho\( \gamma-32P\)ATP\(_1\) complexes were pre-formed by combining \( [\gamma-32P]ATP \) with excess Rho so that on average there was 0.3 \( [\gamma-32P]ATP \) molecule per Rho hexamer. The Rho\( [\gamma-32P]ATP \) complex was formed in a chemical quench apparatus with a solution of poly(C) RNA (required for catalysis) plus a large excess (final concentration, 1–10 mM) of Mg\(_2\)Cl\(_2\) complex of unlabeled nucleotide. The complex had two forms of interest ("chase"). The extent and rate of \([\gamma-32P]ATP \) hydrolysis were determined by removing the reaction after various times (5 ms–30 s) and measuring the amount of product \( 32P \) (see "Experimental Procedures"). The rates of the chemistry step for \( [\gamma-32P]ATP \) hydrolysis and of other events in the catalytic cycle were obtained from simulations of the data using KINSIM (see "Experimental Procedures" and Table I) (14), employing a model that is described below.

Promotion by an ATP Chase—When a high concentration of ATP (2–20 mM) is included with the RNA that is mixed with Rho\( [\gamma-32P]ATP \) complexes, the conditions are closest to the normal in vivo functioning of Rho. All of the vacant catalytic
sulfite rapidly fill with ATP, and RNA binding permits $V_{\text{max}}$ hydrolysis. 20–30% of the total [γ-32P]ATP is hydrolyzed in a burst by 10 ms after mixing, and more than two-thirds is turned over by 200 ms (Fig. 1A). A model that successfully simulates the data is presented in Scheme 1 (also see “Experimental Procedures”). According to this model, the substoichiometric [γ-32P]ATP that is initially bound to Rho is hydrolyzed when, as previously concluded (3, 12), the three active sites fire sequentially. A preexisting asymmetry in the Rho hexamer and/or asymmetry conferred by RNA binding differentiates among the three Rho active sites, defining sites 1, 2, and 3 and resulting in their ordered firing. [γ-32P]ATP and initial RNA binding are at random with respect to one another; hence, one-third of the [γ-32P]ATP is in each of the three sites. The chemistry step of the catalytic cycle is fast (≥300 s⁻¹), so up to one-third of the total [γ-32P]ATP (which is in site 1) is hydrolyzed rapidly (burst) and is found as product 32P at the earliest experimental time points (5–10 ms) (Fig. 1 and Table I). For [γ-32P]ATP molecules initially bound in Rho active site 2 or 3, one and two complete catalytic cycles, respectively, precede the hydrolysis of the labeled substrate molecule. In the simulations, the same steps in each of the three catalytic cycles have the same rates. [γ-32P]ATP can dissociate at any time from Rho (at 3 s⁻¹). Its rebinding is prevented by the high concentration of non-radioactive chase ATP. The steady-state hydrolysis rate usually found for Rho is 30 s⁻¹; however, rates of ~10 s⁻¹ were obtained from modeling.

No Chase—As previously reported (3), when only one of the three ATP binding sites of Rho is occupied and RNA is added in the absence of chase nucleotide, hydrolysis of the bound [γ-32P]ATP differs in two ways from the results with the ATP chase. 1) The rate of catalysis is slow, and 2) there is essentially no burst (Fig. 1A). This result was general for NTPs as demonstrated by experiments in which [γ-32P]ATP was the substrate (Fig. 1, filled triangles).

Although the data when there is no chase appear to be fit by a first-order equation (as in Ref. 3), greater complexity is probably involved. First, some degree of dissociation and rebinding of [γ-32P]ATP during the experiment is expected to occur and

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**Table I.**

| Chase compound | Rate of chemistry step | Burst magnitude |
|----------------|-----------------------|-----------------|
| Promoting compounds | | |
| ATP | ≥300 | 0.25 |
| CTP | ≥300 | 0.25 |
| UTP | 100–300 | 0.25 |
| GTP | 300 | 0.25 |
| Adenine arabinoside-5’-triphosphate | 150–300 | 0.30 |
| 2’-deoxy-ATP | 150–300 | 0.33 |
| 3’-deoxy-ATP | 150–300 | 0.25 |
| ddATP | 300 | 0.15 |
| Non-promoting compounds | | |
| None | 3.2–3.6 | 0.03 |
| CDP | 2.8 | 0.03 |
| AMP-PNP | 3.5 | 0.05 |
| AMP-PCP | 4–5 | 0.05 |
| ADP + P | 1.6 | 0.04 |
| Slowly promoting compounds | | |
| ATP-S | 100 | 0.12 |
| PPP | 60–80 | 0.18 |
| PP | 70–100 | 0.14 |
| PNP | 80–120 | 0.20 |
| ATP | 45 | 0.13 |
| AMP-CPP | 100 | 0.25 |

* A [γ-32P]ATP-rebinding rate of 0.02 s⁻¹ was included in the simulation.
must be modeled. Second, hydrolysis of most of the \( [\gamma^{\text{32P}}]\text{ATP} \) (Fig. 1B and data not shown) requires that \( [\gamma^{\text{32P}}]\text{ATP} \) initially bound in any Rho site eventually can undergo catalysis. With the goal of a single kinetic model that with appropriate rate choices successfully simulates all of the experimental results, the ordered sequential model described above for an ATP chase was applied to this situation with no chase. Two inconsistencies were found. (i) Because there is no substrate or other molecule in two of the active sites, hydrolysis of the \( [\gamma^{\text{32P}}]\text{ATP} \) molecules bound in enzyme sites 2 and 3 cannot be preceded by any catalytic cycles. Although it could reasonably be thought that this situation precludes hydrolysis of \( [\gamma^{\text{32P}}]\text{ATP} \) initially bound in sites 2 and 3, simulations show that hydrolysis by site 1 alone, including hydrolyses following dissociation and random rebinding of \( [\gamma^{\text{32P}}]\text{ATP} \) from other sites, would produce far less product \( ^{32}\text{P} \) than was observed (Fig. 1B, dashed line). Therefore, it is clear that the enzyme must be able to hydrolyze \( [\gamma^{\text{32P}}]\text{ATP} \) initially bound in site 2 or 3 without catalysis in the preceding site(s). Stated another way, enzyme with site 2 or 3 occupied must become like enzyme with \( [\gamma^{\text{32P}}]\text{ATP} \) in site 1 where RNA binding is followed by slow (because two active sites are empty) catalysis. Branches are necessary in the kinetic pathway for these proposed additional first-order events involving enzyme with \( [\gamma^{\text{32P}}]\text{ATP} \) site 2 or 3 (see Scheme 2). Simulations indicate that the rates of such first-order steps are in the range of 2.2–2.4 \( \text{s}^{-1} \). (ii) The second inconsistency when the model for an ATP chase is used for the no-chase situation is that, without energy input from previous catalytic cycles, ordered sequential active site firing must become random. In enzyme molecules with \( [\gamma^{\text{32P}}]\text{ATP} \) bound in site 1, site 1 fires first but enzyme molecules with \( [\gamma^{\text{32P}}]\text{ATP} \) in site 2 or 3 must now behave similarly to one another with a first-order step followed by slow catalysis. When the ATP chase model is modified to accommodate these two inconsistencies (Schemes 2 and 3), the data are closely fit (Fig. 1B). As previously found (3), the rate of the chemistry step of \( [\gamma^{\text{32P}}]\text{ATP} \) hydrolysis is slow (3.2–3.6 \( \text{s}^{-1} \)) (Table I). \( [\gamma^{\text{32P}}]\text{ATP} \) dissociation was at 3 \( \text{s}^{-1} \), and its rebinding was at 0.02 \( \text{s}^{-1} \).

In the absence of a chase compound, no burst was found. Only 3–4% of the radioactivity initially in \( [\gamma^{\text{32P}}]\text{ATP} \) was detected as \( ^{32}\text{P} \). This quantity is of the same magnitude as the background \( ^{32}\text{P} \) in the \( [\gamma^{\text{32P}}]\text{ATP} \) and is not considered significant. A burst of product formation is expected from those Rho hexamers that have all three catalytic sites filled with \( [\gamma^{\text{32P}}]\text{ATP} \); however, under the experimental conditions of 0.1 \( [\gamma^{\text{32P}}]\text{ATP} \) per active site, only 0.1% of the total Rho will have 3 molecules of \( [\gamma^{\text{32P}}]\text{ATP} \) bound and only one of those three bound \( [\gamma^{\text{32P}}]\text{ATP} \) molecules will be hydrolyzed in the burst.

To summarize, in the absence of a chase nucleotide, the kinetics of hydrolysis of a single \( [\gamma^{\text{32P}}]\text{ATP} \) molecule bound to Rho are not only slower than when ATP is saturating but the three Rho active sites no longer necessarily act sequentially. An additional first-order step is required in the enzyme mechanism to permit hydrolysis by enzyme molecules with \( [\gamma^{\text{32P}}]\text{ATP} \) in site 2 or 3.

**Other Promoters**—ATP analogs and the related compounds that we tested to determine the features of a molecule that are important for promotion fell into three classes: 1) those that behaved like ATP; 2) those that enhanced the rate of hydrolysis of \( [\gamma^{\text{32P}}]\text{ATP} \) but not as well as ATP and with a smaller extent of initial hydrolysis; and 3) those that did not promote the hydrolysis of \( [\gamma^{\text{32P}}]\text{ATP} \) or show significant initial \( ^{32}\text{P} \) product formation. Nucleoside monophosphates were not tried since previous work established that AMP binds poorly (10).

**ATP-like Promotion**—Chase nucleotides CTP, GTP (Fig. 2A, filled circles), and UTP, all known Rho substrates (15), behaved like ATP in promoting the hydrolysis rate of \( [\gamma^{\text{32P}}]\text{ATP} \) in Rho\( [\gamma^{\text{32P}}]\text{ATP} \) complexes in the presence of poly(C) and showed an elevated initial product level (burst) (Table I). Among known NTP substrates, the features of the base portion of the nucleotide thus are not critical to promotion. ATP analogs varied in the sugar, 2′- and 3′-dATP, ddATP (Fig. 2B, filled circles),
and adenine arabinoside-5'-triphosphate, which are good Rho substrates, also behaved like ATP (Table I). Although Richardson and Conaway (15) reported that ddATP does not support the release of RNA from ternary transcription complexes, we found from steady-state time course experiments monitored by TLC (see “Experimental Procedures”) that it is hydrolyzed at approximately half the rate of ATP. Thus, the hydroxylation state of the ribose 2'- and 3'-carbons of the nucleotide substrate is also not of great importance in promotion. In the KINSIM model, off-rates of 3 s⁻¹ and catalytic cycle rates of 5–11 s⁻¹ (for the catalytic cycle in site 1 preceding chemistry in site 2 and the cycles in sites 1 and 2 preceding chemistry in site 3) accommodated the data with the exception of 3'-deoxy-ATP where the catalytic cycle was 3 s⁻¹.

Non-promoting Nucleotides—Hydrolysis products ADP and CDP used as chase agents at ~30× Kᵢ were ineffective. The results resembled those obtained in the absence of chase with no rate enhancement or burst (Table I), indicating the importance of the γ-phosphoryl group to promotion. The inclusion of 1 mM Pᵢ with a 1 mM ADP chase did not change the results (Fig. 2A, triangles). This finding is not surprising because the Kᵢ for Pᵢ is ~30 mM (10), so at 1 mM there would be little Pᵢ bound. The use of significantly higher concentrations of Pᵢ and accompanying Mg²⁺ led to the formation of a precipitate, so the effect of the simultaneous presence in Rho active sites of both ATP hydrolysis products is not known. Non-hydrolyzable ATP analogs AMP-PNP (Fig. 2B, triangles) and AMP-PCP (Fig. 2B, open circles) also failed both to promote hydrolysis and to show a significant burst (Fig. 2B and Table I). In KINSIM models, the rates of catalytic cycles preceding chemistry in sites 2 and 3 were set to zero as was done above in the absence of chase but other parameters were close to those for promoting nucleotides. Ligand off-rates were 1.5 s⁻¹, and the added first-order step for enzyme molecules with [γ⁻³²P]ATP in site 2 or 3 was 0.5–1.3 s⁻¹. The chemistry step of the catalytic cycle was again slow (1.6–5.0 s⁻¹) (Table I).

Slower Promotion—Several ATP analogs and related compounds used as chase molecules produced appreciable but lower initial bursts than did ATP and also showed significant rate enhancements during subsequent catalysis. ATPγS, ATP, AMP-CP, PP, PPP, and PNP all behaved in this manner (Fig. 3). The most important contributor to this behavior is in an increase in the rate of the chemistry step for [γ⁻³²P]ATP hydrolysis, modeled by KINSIM as 45–120 s⁻¹ (Table I).

Kᵢ values were obtained for many of the chase compounds by ultrafiltration measurements in competition experiments where [γ⁻³²P]ATP was initially bound (see “Experimental Pro-
Conformations in which product formation. Again, the enzyme must be able to achieve this, because slower chemistry permits more \([\gamma^{32P}]ATP\) dissociation from site 1 prior to hydrolysis. Optimal KINSIM simulations for the \([\gamma^{32P}]ATP\) dissociation rate for this class of compounds were similar to those obtained in other experiments of this type (from 1.5 to 4 s\(^{-1}\)).

The case of ATP\(\gamma S\) as chase is illustrative. Although the results come close to the promotion achieved using ATP as chase (Fig. 3C), experiments carried out side-by-side using as close to the same materials as possible consistently showed less initial \(32P\), product and slower promotion. ATP\(\gamma S\) has been previously characterized as a Rho substrate (3, 16). It is hydrolyzed at 23 °C in the presence of poly(C) at \(t = 1\) s \(^{-1}\) versus 30 s \(^{-1}\) for ATP. KINSIM modeling of the results with ATP\(\gamma S\) as chase failed to match the data when its hydrolysis at \(t = 1\) s \(^{-1}\) was forced to precede that of \([\gamma^{32P}]ATP\) in Rho sites 2 and 3 (ordered sequential mechanism). If this were the mechanism, a plateau after hydrolysis of approximately one-third of the total \([\gamma^{32P}]ATP\) is expected as the slow hydrolysis of ATP\(\gamma S\) permits time for \([\gamma^{32P}]ATP\) dissociation at 3 s \(^{-1}\). However, the data could be well fit (Fig. 3C) by a 100-s \(^{-1}\) \([\gamma^{32P}]ATP\) chemistry step together with a 3–4-s \(^{-1}\) first-order step preceding \([\gamma^{32P}]ATP\) hydrolysis in active site 2 or 3. Thus, ATP\(\gamma S\) hydrolysis at \(t = 1\) s \(^{-1}\) is too slow to be a significant step in ATP\(\gamma S\)-promoted \(32P\) production from \([\gamma^{32P}]ATP\). ATP\(\gamma S\) must, like the other slow promoters that are not appreciably hydrolyzed, enable the enzyme active sites to achieve a conformation close to that when ATP is present in all active sites. A comparison of the results of \([\gamma^{32P}]ATP\) hydrolysis with an ATP\(\gamma S\) chase and \([35S]ATP\)\(\gamma S\) hydrolysis with ATP chase revealed, as expected, that ATP does not promote fast hydrolysis of ATP\(\gamma S\) (data not shown), consistent with a slow chemistry step for this analog (17).

**DISCUSSION**

**New Enzyme Behavior under Substoichiometric Substrate Conditions**—When poly(C) is added to Rho that has ATP bound, ATP hydrolysis is 30-fold faster when all three active sites contain ATP than it is when only one site is occupied (3). The kinetics and extent of \([\gamma^{32P}]ATP\) hydrolysis with no chase, non-promoting chase, or with slowly promoting chase compounds reinforce the concept introduced by the finding of ordered sequential catalysis among its three active sites that the Rho hexamer is functionally asymmetric and illuminate two additional facets of Rho behavior. First, ATP or other strong promoter binding in all of the active sites permits the enzyme to adopt optimal active site configurations and results in ordered sequential substrate hydrolysis with fast chemistry steps (Schemes 1 and 3 and Table I). The slower promoters allow Rho active sites to achieve configurations competent for chemistry but at lower rates. Second, when \([\gamma^{32P}]ATP\) or other strong promoter occupies only active site 2 or 3, the enzyme can undergo a slow conformation change that essentially converts it to the form with \([\gamma^{32P}]ATP\) bound in site 1 and then catalysis can occur (Scheme 2). ATP-saturated enzyme can probably also undergo these conformation changes (Scheme 3), but they are so slow relative to the steps of the ordered sequential firing pathway that their contribution to hydrolysis is minimal.

**TABLE II**

Catalytic Cooperativity in E. coli Rho Hexamers

| Chase compound | \(K_0\) | \(\mu M\) |
|----------------|--------|----------|
| **Promoting compounds** | | |
| ATP | 0.2–0.5 | |
| CTP | 2.2–2.5 | |
| 2'-dATP | 1 ± 0.1 | |
| **Non-promoting compounds** | | |
| AMP-PNP | 4.8 ± 0.1 | |
| AMP-CP | 2.5 ± 0.1 | |
| ADP + P\(_i\) | 27 (ADP\(^\mu\)) | |
| **Slowly promoting compounds** | | |
| PPP | 160 ± 16 | |
| PP | 15 ± 2 | |
| PNP | 35 ± 4 | |
| ATP | 3.9 ± 0.4 | |
| AMP-CPP | 43 ± 3 | |

* Determined using Rho E155K in buffer containing 50 mM KCl instead of potassium glutamate (10).

The values obtained are given in Table II and show that, at 1 mM final concentrations, Rho active sites will be saturated. The possibility that slower on-rates contributed to the slower promotion was tested with PP\(_i\), by repeating the experiment at a 4-fold higher MgPP\(_i\) concentration. No change was seen in the results (Fig. 3B, open versus filled triangles), indicating that the on-rate was not limiting.

**Slower Promoters Are Not Significantly Hydrolyzed**—The slower promotion of ATP hydrolysis by these compounds prompted us to determine whether they are substrates for Rho. We found very slow hydrolysis of \([\gamma^{32P}]PPi\) at 37 °C of 0.5 \(\times 10^{-4}\) s\(^{-1}\) measured by quantitation of product \(32P\), following TLC of reaction samples (see “Experimental Procedures”). Including a small amount (10 \(\mu M\)) of ATP to more closely mimic the conditions of the chase experiments yielded a similar result (data not shown). This extremely slow PP\(_i\), hydrolysis rate indicates that, although PP\(_i\) is able to promote ATP hydrolysis, its own hydrolysis is not part of the process. An analysis of PPP, found no evidence for its hydrolysis by Rho after 60 h at room temperature (see “Experimental Procedures”), whereas AMP-CPP was a slow substrate with an estimated turnover rate on the order of 0.1–1 s\(^{-1}\). AMP-CP product was visible after 1 h of reaction at room temperature with complete hydrolysis by 4 h under conditions where the same initial concentration of ATP was fully hydrolyzed by ~5 min (see “Experimental Procedures”). Under similar conditions, ATPP was a very slow substrate, requiring overnight incubation for ~50% hydrolysis (analyzed by TLC). These results indicate that these compounds promote the rate of \([\gamma^{32P}]ATP\) hydrolysis without being hydrolyzed over the time period of promotion. This conclusion is supported by the similar behavior of PNP, a non-hydrolyzable PP\(_i\) analog (Table I and Fig. 3B).

As in the situation with no chase, more than one-third of the \([\gamma^{32P}]ATP\) is eventually hydrolyzed. In these slower promoter chases, this occurs before 400 ms (Fig. 3). Unlike the situation with no chase, re-binding and hydrolysis of dissociated \([\gamma^{32P}]ATP\) cannot account for a significant portion of this product formation. Again, the enzyme must be able to achieve conformations in which \([\gamma^{32P}]ATP\) bound in site 2 or 3 can be hydrolyzed without catalysis in the previous site(s). KINSIM yields rates for these first-order steps of 1–4 s\(^{-1}\).

**Lower Bursts with Slower Promoters**—To explain the lower bursts of \([\gamma^{32P}]ATP\) hydrolysis found with the slower promoters, we reasoned as follows. Essentially all of the \([\gamma^{32P}]ATP\) in these experiments is initially bound at random to Rho active sites. As in the case where there is an ATP chase, the hydrolysis of one-third of the total \([\gamma^{32P}]ATP\) in a burst can be understood as the hydrolysis of \([\gamma^{32P}]ATP\) bound in Rho site 1, for which there is no required preceding catalytic cycle. However, in the case of the slower promoters, clearly less than one-third of the total \([\gamma^{32P}]ATP\) is hydrolyzed in a burst but more is hydrolyzed than with non-promoting chase compounds (Figs. 2 and 3 and Table I). The burst is smaller than with an ATP-like promoter, because slower chemistry permits more \([\gamma^{32P}]ATP\) dissociation from site 1 prior to hydrolysis. Optimal KINSIM simulations for the \([\gamma^{32P}]ATP\) dissociation rate for this class of compounds were similar to those obtained in other experiments of this type (from 1.5 to 4 s\(^{-1}\)).

The case of ATP\(\gamma S\) as chase is illustrative. Although the results come close to the promotion achieved using ATP as chase (Fig. 3C), experiments carried out side-by-side using as close to the same materials as possible consistently showed less initial \(32P\), product and slower promotion. ATP\(\gamma S\) has been previously characterized as a Rho substrate (3, 16). It is hydrolyzed at 23 °C in the presence of poly(C) at \(t = 1\) s \(^{-1}\) versus 30 s \(^{-1}\) for ATP. KINSIM modeling of the results with ATP\(\gamma S\) as chase failed to match the data when its hydrolysis at \(t = 1\) s \(^{-1}\) was forced to precede that of \([\gamma^{32P}]ATP\) in Rho sites 2 and 3 (ordered sequential mechanism). If this were the mechanism, a plateau after hydrolysis of approximately one-third of the total \([\gamma^{32P}]ATP\) is expected as the slow hydrolysis of ATP\(\gamma S\) permits time for \([\gamma^{32P}]ATP\) dissociation at 3 s \(^{-1}\). However, the data could be well fit (Fig. 3C) by a 100-s \(^{-1}\) \([\gamma^{32P}]ATP\) chemistry step together with a 3–4-s \(^{-1}\) first-order step preceding \([\gamma^{32P}]ATP\) hydrolysis in active site 2 or 3. Thus, ATP\(\gamma S\) hydrolysis at \(t = 1\) s \(^{-1}\) is too slow to be a significant step in ATP\(\gamma S\)-promoted \(32P\) production from \([\gamma^{32P}]ATP\). ATP\(\gamma S\) must, like the other slow promoters that are not appreciably hydrolyzed, enable the enzyme active sites to achieve a conformation close to that when ATP is present in all active sites. A comparison of the results of \([\gamma^{32P}]ATP\) hydrolysis with an ATP\(\gamma S\) chase and \([35S]ATP\)\(\gamma S\) hydrolysis with ATP chase revealed, as expected, that ATP does not promote fast hydrolysis of ATP\(\gamma S\) (data not shown), consistent with a slow chemistry step for this analog (17).
These kinetic branches only achieve visibility when chemistry is slow. When there is no chase or with chase compounds that are not hydrolyzed, the slow branches are the only routes to hydrolysis of $[\gamma^32P]ATP$ initially bound in site 2 or 3. Thermodynamic considerations mandate that hydrolysis by enzymes with ATP in site 2 or 3 without catalysis in preceding sites must occur in a random, rather than an ordered sequence, because there is no energy input from preceding catalysis that can impose order.

**Modeling the Data**—We constructed and tested kinetic models for our results with the goal of a single model for all of the data from similar experiments. This goal was achieved. We are aware of the limitations of modeling and attempted to determine the sensitivity of the rates in the best simulations. In general, optimal rates could be varied by less than a factor of two without producing an obviously poorer fit to the data. It is satisfying that similar values were obtained from modeling different data sets for the off-rate for $[\gamma^32P]ATP$ and for the slow first-order step permitting catalysis by enzyme with $[\gamma^32P]ATP$ in site 2 or 3. The rapid mix/chemical quench results are successfully simulated using the models shown in Schemes 1 and 2 and their composite shown in Scheme 3. (i) When there is promotion of the hydrolysis of a single bound radiolabeled ATP molecule per hexamer, the hydrolysis rate is consistent with random labeled ATP and RNA binding followed by ordered sequential active site firing. An alternative model in which RNA binds preferentially to the Rho subunit that contains $[\gamma^32P]ATP$ did not fit the data, producing simulations with faster and larger amplitude hydrolysis than was actually seen (data not shown). (ii) When there is no chase nucleotide present, a non-promoting chase nucleotide, or a slowly promoting chase compound, chemistry is slower, permitting the slow first-order step to emerge, and ordered sequential activity becomes random. Stated differently, enzyme molecules with $[\gamma^32P]ATP$ in site 2 or site 3 in the absence of catalysis at normally preceding sites can undergo a slow first-order event that enables them to perform catalysis.

Thus, two conformation changes are implicit in the model. One occurs upon the binding of ATP or other strong promoter molecule to an active site. This conclusion is consistent with the intrinsic Rho fluorescence changes upon ATP binding studied by Jeong et al. (18). If all of the active sites undergo this conformation change, catalysis is fast and ordered sequential. In the KINSIM model, this is reflected in the rate of the chemistry step. If only one site has ATP bound, when it is site 1, slow chemistry (because the other two active sites are empty) follows. If the molecule of ATP is bound in site 2 or 3, an additional first-order isomerization step is needed prior to a slow chemistry step. Different chase molecules lead to very different chemistry step rates (Table I), whereas the slow first-order step rate does not vary greatly. The slow first-order step could be a protein conformation change, or it might involve the repositioning of bound RNA in relation to an empty active site to a more productive configuration with respect to an active site that is filled with $[\gamma^32P]ATP$.

One notable aspect of the KINSIM model for the ATP chase data (Fig. 1) is the $10^{-1}$ rate that provided the best fit for the overall catalytic cycle. Previous work (3, 12) led us to expect a rate of $30 \text{ s}^{-1}$. It is not clear whether this 3-fold rate difference is significant. However, models employing a catalytic cycle rate of $30 \text{ s}^{-1}$ gave a poor fit to the data. Those fits could be improved by reducing the rate of the chemistry step from 300 to $\sim 80 \text{ s}^{-1}$ and by increasing the ATP dissociation rate from 3 to $8 \text{ s}^{-1}$, but these values deviate from our previous findings. Intermediate values (e.g., $20 \text{ s}^{-1}$) for the catalytic rate provided reasonable fits with corresponding lesser adjustments of the dissociation and chemistry steps. Although this rate discrepancy might reflect an enzymatic property that we do not yet appreciate, it is more likely that it is due to minor differences in mixing order and ligand concentrations in the various experiments compared. It is clear that the kinetics of ATP hydrolysis by Rho depend on the degree of saturation of Rho with ATP (3). There may be other variations of rates that depend, for example, on ligand binding order. It is clear that we do not yet know the details and order of events at all of the catalytic and binding sites on Rho. Much of our current work is directed toward answering these questions.

**Substrate Characteristics Needed for Catalytic Cooperativity**—A goal of this work was to determine the features of the ATP molecule that are needed for the increased catalytic rate under saturating substrate conditions. Several results illustrate the importance of the phosphoryl chain of the nucleotide relative to that of the base and sugar. 1) There is significant rate enhancement by PP$\_6$, PPP$\_6$, and PNP, which have no base or sugar. 2) Nucleoside triphosphates with any of the four conventional bases are effective promoters. There is no base specificity. 3) The length of the phosphoryl chain of a nucleotide is critical. Nucleoside diphosphates are ineffective, and the tetraphosphate ATP$\_4$ is not as efficient as nucleoside triphosphates.

Consideration of the Rho crystal structure with bound AMP-PNP (19) is useful in interpreting the ATP analog results, although the presence of six molecules of AMP-PNP per hexamer (in contrast to the three MgATP binding sites that we reproducibly find (10, 12)) and the curious absence of Mg$^{2+}$ in the structure suggest caution in drawing conclusions. The adenosine base of AMP-PNP is sandwiched between the side chains of Phe$^{355}$ and Met$^{146}$. The hydrophobic interactions involved are not base-specific, consistent with the promotion ability of nucleoside triphosphates with any of the normal bases (Table I). The ribose hydroxyl groups of AMP-PNP are exposed at subunit interfaces that are splayed open in the available structure but that must close to accomplish catalysis. Our results suggest that specific interactions between the protein and these hydroxyl groups are not of major significance even in a closed protein, because 2'$^\text{-dATP}$, 3'$^\text{-dATP}$, dATP, and adenosine arabinoside-5'$^\text{-triphosphate}$ are substrates and promoters (Fig. 2 and Table I). The phosphoryl group interactions with Rho seen in the co-crystal structure are not what are typically found in proteins with Walker A- and B-sequences, perhaps because of the open configuration at the subunit interfaces (19). Our finding that AMP-PNP is not a promoter further suggests that its phosphoryl groups are not able to interact with the protein in the same way as those of ATP. The subtle structural differences between PNP and PP$\_6$, the interaction of AMP-PNP with Mg$^{2+}$, or the lower acidity of the terminal phosphoryl group of AMP-PNP compared with ATP (20) may be contributing factors. Although AMP-PNP does not promote $[\gamma^32P]ATP$ hydrolysis, the smaller PNP molecule is a slow promoter (Fig. 3 and Table I). These results indicate that base and ribose contacts with Rho that are available to AMP-PNP thwart productive phosphoryl group interactions with the protein. The fact that AMP-PNP behaves like ADP with respect to promotion (Fig. 2 and Table I) is consistent with the repeated finding that this analog resembles ADP rather than ATP (18, 21).

Consideration of PNP also illustrates the finding that significant rate enhancement does not require coordinated hydrolysis of a phosphodiester bond. PNP and also PP$\_6$ and PPP$\_6$ are hydrolyzed at an insignificant rate but are nevertheless slow promoters. These compounds must be able to bind in the place normally occupied by the phosphoryl chain of ATP and interact with moderate efficiency. However, the best promotion is achieved when a hydrolyzable nucleotide is used.
Comparison with Other Enzymes—Catalytic cooperativity in the absence of binding cooperativity, as seen here, has been discussed (22) and is documented, for example, for yeast AMP deaminase, a homotetrameric protein (23, 24). Substrate AMP binds with equal affinity to all of the catalytic sites, and in the absence of regulatory ligands, the catalytic rate increases as sites are filled.

F₁ ATPase is perhaps the best-known enzyme that exhibits catalytic cooperativity. Its αβγδε structure is reminiscent of Rho. The α- and β-subunits are quite similar and are assembled as a planar hexamer (25), the probable structure for Rho once long RNA is bound (26). The ATP binding portion of the β-subunits where the active sites are located is very similar to the ATP binding portion of Rho, both in amino acid sequence and in structure (19, 27, 28). The enzymes are different in that ATP binding to F₁, unlike its binding to Rho, exhibits negative and in structure (19, 27, 28). The enzymes are different in that ATP binding to F₁, unlike its binding to Rho, exhibits negative cooperation (e.g. Ref. 29). F₁ has been well characterized as carrying out slow net ATP hydrolysis when a single molecule of ATP is bound and 10⁶-fold faster catalysis when ATP is saturating (2), a far larger difference than is seen with Rho. For F₁, ATP binding in additional catalytic sites promotes product release from the originally occupied site, whereas for Rho, additional substrate binding promotes the chemistry step of catalysis. F₁ is similar to Rho in that substrates that efficiently promote catalytic cooperativity include other hydrolyzable nucleotides (1). Non-hydrolyzable ATP analogs such as AMP-PNP and ADP, however, behave differently with F₁, leading to intermediate promotion (1), and additional studies with F₁ (e.g. Ref. 30) suggest greater complexity with this enzyme. These disparities are likely to reflect differences in the details of how the two different proteins interact with nucleotides and their analogs and how the two proteins respond to such interactions.

Similar to the situation with Rho, different degrees of enzyme conformation change depending on whether substrate or a substrate analog binds in the active site have been previously reported as with Ca²⁺-ATPase, for example (31). Other enzymes, such as tryptophan synthase (reviewed in Ref. 32; see also Ref. 33), provide additional examples of conformational change depending on whether substrate or substrate analog binding in another subunit.

Summary—The β- and γ-phosphoryl groups of ATP play a key role in promoting Vₘₐₓ ATP hydrolysis by Rho and are responsible for promoting catalytic cooperativity among the Rho active sites. When a single ATP molecule is bound per Rho hexamer, a slow first-order step that precedes chemistry can occur in the presence of RNA, enabling ATP hydrolysis in any active site. In the absence of saturating hydrolyzable substrate, the rate of catalysis by Rho depends to a significant extent on this slow first-order event, which then emerges together with the chemistry step to kinetic prominence. The first-order step could be a protein conformation change or a rearrangement of Rho-bound RNA.

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