Active Site Occupancy Required for Catalytic Cooperativity by *Escherichia coli* Transcription Termination Factor Rho*

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*Escherichia coli* transcription termination factor Rho exhibits the phenomenon of catalytic cooperativity. The catalytic rate per site is 30-fold faster when all three sites are filled with substrate ATP than when only a single site is occupied (Stitt, B. L., and Xu, Y. (1998) *J. Biol. Chem.* 273, 26477–26486). Experiments presented here investigate whether all three active sites must be filled or whether only two occupied sites are required for catalytic cooperativity. The results indicate that all three Rho catalytic sites must be filled with substrate to achieve the enhanced catalytic rate, both in pre-steady-state and in steady-state hydrolysis. They further suggest that, once the enzyme is saturated with ATP, a $V_{\text{max}}$ enzyme conformation is achieved that is stable for at least three catalytic cycles.

In some enzymes that have multiple active sites, the rate constants for events at individual sites increase as substrate occupies more of the catalytic sites. Such catalytic cooperativity has been well documented for mitochondrial F$_1$-ATPase/synthase, an enzyme with three active sites in which there is a dramatic 10$^6$-fold increase in the hydrolysis rate between singly occupied and substrate-saturated enzyme (1, 2). This cooperativity is important in thinking about energetics. For F$_1$, ATP synthase, energy input is associated with ATP release rather than formation of the phosphoanhydride bond and individual active sites are utilized in an ordered sequential fashion (reviewed in Ref. 3).

Although, in the case of F$_1$, catalytic cooperativity is fundamental to the enzyme mechanism, it is not yet clear how widespread catalytic cooperativity is among other proteins. There are examples in which there is strong suggestive evidence such as AMP deaminase, a tetrameric enzyme, that exhibits concave-up Lineweaver-Burk plots, behavior that is consistent with catalytic cooperativity (4, 5). In the case of the trimeric mammalian purine nucleoside phosphorylase, the binding stoichiometry of one exhibited by a transition state analog is consistent with catalytic cooperativity (6).

*Escherichia coli* transcription termination factor Rho also exhibits catalytic cooperativity (7). Rho is a homohexameric helicase with three demonstrated active sites that catalyze ATP hydrolysis in an RNA-dependent fashion (reviewed in Ref. 8). Rho uses the energy from ATP hydrolysis to travel $5' \rightarrow 3'$ along the nascent RNA of ternary transcription complexes and, at appropriate sites, terminate transcription. RNA-dependent ATP hydrolysis by Rho is routinely studied in a simplified system that requires only Rho, RNA, and MgATP.

Our work to date has demonstrated several points that are important in understanding how the energy from ATP hydrolysis is used by Rho for directional movement along RNA. (i) During hydrolysis of ATP by Rho, the $\gamma$-phosphoryl group of ATP is transferred directly and irreversibly to water (9, 10). This point was demonstrated through use of an ATP analog with a chiral $\gamma$-phosphoryl group, which underwent inversion of phosphate stereochemistry upon hydrolysis. Oxygen exchange experiments (10) revealed only a single water-derived oxygen in the product phosphate. These findings eliminate the possibility of a phosphorylated enzyme or RNA intermediate during catalysis and demand that the hydrolysis energy be used to drive a cycle of enzyme conformation changes. Kinetic mechanisms developed from these results are consistent with the involvement of RNA binding and release during such conformation changes (10).

(ii) Pre-steady-state kinetics experiments show that the enzyme steady-state is preceded by a hydrolysis burst. These results indicate that the chemistry step of the catalytic cycle is rapid and is followed by at least one slow rate-determining step in each cycle. The pre-steady-state behavior can be fit by models that include estimates for all rate constants (11).

(iii) Catalytic events among the three Rho active sites proceed in an ordered sequential pattern. This conclusion was most clearly established by two rapid mix/chemical quench experiments (7, 12). When Rho is mixed with excess $[\gamma^{32}\text{P}]\text{ATP}$ plus RNA, a hydrolysis burst of one molecule of $[\gamma^{32}\text{P}]\text{ATP}$ is found per hexamer. When Rho with a saturating amount of unlabeled ATP is mixed with RNA plus excess $[\gamma^{32}\text{P}]\text{ATP}$, a delay in the hydrolysis of $[\gamma^{32}\text{P}]\text{ATP}$ is seen that corresponds to the time required for the hydrolysis of three ATP molecules per hexamer. The sequential hydrolysis pattern readily fits into models for directional movement of Rho along RNA.

(iv) Rho exhibits catalytic cooperativity. This finding is in accord with the previous observations of burst kinetics together with ordered sequential active site firing. The active sites must communicate to achieve these hydrolysis characteristics. In addition, the rate of the chemistry step of the catalytic cycle is increased when the ATP substrate is saturating. The steady-state ATP hydrolysis rate is $\sim$30-fold slower when a single ATP molecule is bound to Rho than when all three active sites are filled (7).

Many of the above conclusions derive from pre-steady-state kinetics measurements, but it is the enzyme steady state that is of greatest interest. With respect to catalytic cooperativity, one important question concerns whether all three catalytic sites must be occupied to achieve $V_{\text{max}}$ catalysis. The precise kinetic, mechanistic, and structural details of catalytic coop-
erativity in F₁ are the ongoing subject of intense study. Rho also provides a valuable model system. The enzyme is stable and of simpler structure than F₁, and all of its nucleotide binding sites are catalytic sites. The rate constants for ATP hydrolysis by Rho make it highly amenable to available chemical quench approaches.

In the accompanying paper (11), we examined what features of the ATP substrate are important to achieve the catalytic rate enhancement at saturating substrate concentrations and found that the configuration of the β- and ϒ-phosphoryl groups is key. This work addresses the question of how many of the three Rho active sites must be filled to enhance the hydrolysis rate, whether the activated enzyme state is persistent and kinetically relevant, and whether an ordered sequential catalytic sequence is operative during the steady state.

### EXPERIMENTAL PROCEDURES

**Rho Occupancy Experiments**—In rapid mix/chemical quench experiments similar to those described in the accompanying paper (11), the magnitude of initial [γ-³²P]ATP hydrolysis was measured at various [γ-³²P]ATP concentrations between 0.4 and 9 μM with aging times from 5 to 208 ms. The amount of bound [γ-³²P]ATP was determined by centrifugal ultrafiltration experiments (12). To calculate the proportion of Rho molecules with 0, 1, 2, and 3 sites occupied by [γ-³²P]ATP, the following expressions based on a polynomial expansion were used where x is the overall ratio of [mol [γ-³²P]ATP bound]/[mol Rho hexamer], the probability that one particular Rho active site is occupied is x/3, and the probability that that site is not occupied is (1 – x/3). The calculation utilizes our finding that ATP binds randomly to the three Rho catalytic sites (Table 1).

**Trapping from the Steady State**—Isotope trapping from the steady state followed the general principles discussed by Wilkinson and Rose (13) and Cao et al. (14). Rapid mix/chemical quench experiments were conducted as in Browne et al. (11) except that, to initiate the reaction, Rho from one syringe was mixed with poly(C) + 200 μM [γ-³²P]ATP from the other syringe. Duplicate points with aging times from 10 to 420 ms were carried out. For each aging time, one of the duplicate reactions (~100 μl) was quenched by injection into 400 μl of 10.8 μM unlabeled ATP, 12 mM Mg acetate, 48 mM Tris acetate, pH 8.3 at 25 °C, and 20% (w/v) trichloroacetic acid. The second identical reaction was injected into 240 μl of a rapidly stirred “trap” solution that was 18 μM unlabeled ATP, 20 μM Mg acetate, and 80 μM Tris acetate, pH 8.3 at 25 °C. After 2 s, this reaction was quenched by the addition of 160 μl of 50% trichloroacetic acid and total radioactivity was measured in a 50-μl sample. Product [³²P]P was measured in a second 50-μl sample following charcoal precipitation of adenine nucleotides (11). The amount of hydrolysis (nmol [³²P]/nmol Rho hexamer) was calculated and plotted as described by Cao et al. (14).

**KINSIM** (15) models were used to obtain limits on the P₃ and ADP off-rates consistent with the steady-state trapping data. The numerical integration models used had three consecutive catalytic cycles, each with a chemistry step at 300 s⁻¹ followed by ADP or P₃ dissociation, ATP rebinding at 300 s⁻¹, and a 30 s⁻¹ step to complete the catalytic cycle. The proportion of enzyme with three ATP molecules bound was displayed versus time for various product off-rates and compared with experimental results.

**Isotope Partitioning** (16, 17)—3.3 μM Rho hexamer saturated with 40 μM [γ-³²P]ATP was mixed with poly(C) plus 2 or 20 μM MgATP or with poly(C) plus 20 mM MgADP in a rapid mix/chemical quench apparatus. Samples were quenched at the indicated times after mixing and analyzed for hydrolysis of [γ-³²P]ATP by measurement of [³²P], as described (11). In some experiments, for each time point, the reagents were loaded into the tubings of the apparatus (Update Instruments) that connect the drive syringes to the mixer as described (11) and buffer was loaded into the syringes. This configuration permitted the most similar conditions in experiments with ATP and ADP chases, because the different chase nucleotides could be used for the same time points immediately following one another.

The KINSIM model for predicting [γ-³²P]ATP hydrolysis with different numbers of [γ-³²P]ATP-occupied sites included steps for three sequential catalytic cycles and assumed a chemistry step at 300 s⁻¹ when ATP was saturating followed by the remainder of the catalytic cycle at 30 s⁻¹ and a [γ-³²P]ATP dissociation rate from any species of 1–3 s⁻¹. The rate of the chemistry step was changed to 3 s⁻¹ when the presence of bound ADP was assumed to change the [γ-³²P]ATP hydrolysis rate based on the results in Browne et al. (11).

### RESULTS

**Size of the Initial ATP Hydrolysis Burst**—We have previously shown that the rate of ATP hydrolysis by Rho is enhanced when all three active sites are occupied (7). When Rho saturated with ATP is mixed in a rapid mix/chemical quench apparatus with poly(C) RNA, there is also a burst representing hydrolysis of one molecule of ATP per Rho hexamer. The burst is complete at the earliest measurable time after mixing (~3 ms) (7, 12). However, when a single ATP molecule is initially bound per hexamer, this burst is absent and hydrolysis proceeds ~30-fold more slowly (7, 11). The burst behavior was exploited in experiments in which mixtures with varying ratios of triply, doubly, and singly occupied Rho species were rapidly mixed with RNA and the size of the burst was measured.

The relationship between the magnitude of the burst and the proportions of the variously occupied Rho molecules was then assessed. The fractions of the hexamer population with 0, 1, 2, and 3 molecules of [γ-³²P]ATP bound were calculated assuming random ATP binding to three catalytic sites using a polynomial expansion (see “Experimental Procedures”). The curves in Fig. 1 indicate the expected proportions of variously occupied Rho molecules at different molar ATP/Rho ratios, and the points are the experimental results. Lines representing full burst activity at 1 or 2 ATP molecules per hexamer do not fit the results. The data most closely fit the theoretical curve for which three bound ATP molecules are required. Although in general the points fit the theoretical curve well, a lower burst than expected was found under the condition where ~2.5 ATP molecules per Rho hexamer were bound. The reason for this discrepancy is not known.

**Isotope Partitioning with a Non-promoting Chase**—Because our current model for Rho action envisions catalytic cooperativity as steady-state behavior, we wished to determine whether the catalytically cooperative enzyme configuration induced by full ATP occupancy persists beyond the initial burst phase. Rho with sufficient [γ-³²P]ATP to fill all of the binding sites was
mixed in a chemical quench apparatus with RNA plus a high concentration of ADP, a nucleotide that binds to active sites (10) but that does not itself promote catalytic cooperativity (11). The ADP was treated with hexokinase (11) prior to use to remove possible ATP contamination. If the catalytically cooperative enzyme state is short-lived, the rate of hydrolysis of the three bound labeled ATP molecules is predicted to decrease after hydrolysis of the first one (Fig. 2, long dashed line) or two (Fig. 2, short dashed line) [γ-32P]ATP molecules. In this experiment, Rho is initially fully occupied with [γ-32P]ATP and the 10 mM concentration of MgADP means that ADP will rapidly occupy catalytic sites from which any nucleotide has dissociated. The results are shown in Fig. 2 where they are compared with a similar experiment in which ATP was the chase nucleotide. The two experiments gave indistinguishable results. A burst of one ATP per hexamer was seen followed by hydrolysis of 1.5–2 additional ATP molecules per hexamer. Although there is a low level of ATP hydrolysis of the burst magnitude, it is most closely related to the proportion of Rho that has three molecules of ATP bound. If the requirement for ATP molecules bound in only two active sites, the burst magnitude would be proportional to the sum of the doubly plus triply occupied Rho species, which is clearly not the case (Fig. 1).

The previous experimental results were both obtained under pre-steady-state conditions, so it is possible that they may not apply to the steady-state enzyme (18). To extend the investigation to steady-state catalysis, we determined the average number of ATP molecules bound during steady-state hydrolysis. In rapid mix/chemical quench experiments, enzyme was mixed in a chemical quench apparatus with RNA plus a high concentration of ADP, a nucleotide that binds to active sites (10) but that does not itself promote catalytic cooperativity (11). The ADP was treated with hexokinase (11) prior to use to remove possible ATP contamination. If the catalytically cooperative enzyme state is short-lived, the rate of hydrolysis of the three bound labeled ATP molecules is predicted to decrease after hydrolysis of the first one (Fig. 2, long dashed line) or two (Fig. 2, short dashed line) [γ-32P]ATP molecules. In this experiment, Rho is initially fully occupied with [γ-32P]ATP and the 10 mM concentration of MgADP means that ADP will rapidly occupy catalytic sites from which any nucleotide has dissociated. The results are shown in Fig. 2 where they are compared with a similar experiment in which ATP was the chase nucleotide. The two experiments gave indistinguishable results. A burst of one ATP per hexamer was seen followed by hydrolysis of 1.5–2 additional ATP molecules per hexamer. Although there is a low level of ATP hydrolysis of the burst magnitude, it is most closely related to the proportion of Rho that has three molecules of ATP bound. If the requirement for ATP molecules bound in only two active sites, the burst magnitude would be proportional to the sum of the doubly plus triply occupied Rho species, which is clearly not the case (Fig. 1).

The lower than expected burst sizes at ~2.5 ATP/hexamer (Fig. 1) could be interpreted as indicating more than three ATP binding sites. For example, if the enzyme simultaneously uses six ATP binding sites, it might be that 4, 5, or 6 of these need to be occupied to achieve the full burst magnitude of 1 mol of P/mol hexamer. Indeed, ATP sites on all six identical Rho subunits are occupied in co-crystal structures with nucleic acid oligomers and millimolar levels of the ATP analogs AMP-PNP or adenosine 5′-3-O-(thio)triphosphate (19, 20). However, discrepancies between liganding behavior in solution and in crystals have been previously noted. In the case of the homotrimeric enzyme purine nucleoside phosphorylase, binding in solution of one molecule of a transition-state inhibitor per trimer prevents inhibitor binding at the other two subunits (6), yet three molecules per trimer are bound in crystals (21). Other analyses of Rho have reported evidence for two different binding classes (of three sites each) for adenine nucleotides, but catalytic competency of the sites is less certain (22, 23). Our models are based on measurements that clearly demonstrate three non-interacting ATP binding sites per hexamer that are also active sites (7, 10, 12).

To examine the dynamics of the catalytically cooperative enzyme state, the enzyme was pre-loaded with [γ-32P]ATP plotted against the number of ATP molecules hydrolyzed per molecule of hexamer in the quench. A straight line fit to these data intercepts the y-axis at a value that is equal to the average number of ATP molecules per hexamer trapped from the steady state. This number is three.

**DISCUSSION**

**Number of Bound ATP Molecules Required for Vmax**—From the three experiments that address catalytic cooperativity in Rho, both static and dynamic information were obtained. The initial amount of ATP hydrolyzed (burst magnitude) as the ATP site occupancy is varied reflects the enzyme state at a single point in time and thus is essentially a static measurement. The burst magnitude best supports the conclusion that three filled sites are required for rapid ATP hydrolysis, because it is most closely related to the proportion of Rho that has three molecules of ATP bound. If the requirement were for ATP molecules bound in only two active sites, the burst magnitude would be proportional to the sum of the doubly plus triply occupied Rho species, which is clearly not the case (Fig. 1).

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1 The abbreviations used is: AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate.
and chased with RNA plus a high concentration of ADP. Fig. 2 shows that, after the hydrolysis of one and even two ATP molecules per hexamer and their replacement with ADP, the rate of hydrolysis of the remaining bound ATP is as fast as when a high concentration of ATP is present. These results indicate that, once Rho has acquired the conformation necessary for catalytic cooperativity and $V_{\text{max}}$ catalysis, this conformation is maintained at least for the duration of three catalytic cycles.

Our current model for ordered sequential ATP hydrolysis by Rho (11, 12) is partly based on the results from pre-steady-state catalysis experiments (7, 12). The present steady-state trapping results extend this mechanism to steady-state hydrolysis conditions. All three Rho active sites are filled with ATP during the majority of the catalytic cycle. These results also require that product off-rates are fast, otherwise an average of fewer than three ATP molecules per hexamer would have been found. KINSIM modeling (see “Experimental Procedures”) suggests that ADP and $F_1$ dissociation must each occur at least at 120 s$^{-1}$ to yield the observed steady-state trapping results. Because burst kinetics indicate that both ATP and RNA binding are fast as is chemistry ($>300$ s$^{-1}$ (7)), the step(s) of the catalytic cycle that determine the $30$ s$^{-1} V_{\text{max}}$ hydrolysis rate must involve either RNA dissociation or protein conformation change(s). The rate-limiting step must occur after the dissociation of hydrolysis products and the binding of fresh substrate ATP (Fig. 4).

Comparison with Other Enzymes—$F_1$ ATPase is the best-studied enzyme that exhibits catalytic cooperativity. Although it is a structurally more complex protein than Rho, the major $\alpha$-and $\beta$-subunits of the $\alpha_2\beta_2\gamma_6\delta$ structure are reminiscent of Rho and are assembled as a planar hexamer (24), which may also be the structure adopted by Rho when it is complexed with an RNA polymer (25). The similarities between Rho and $F_1$ extend to the number, structure, and amino acid sequence of the ATP active sites (19, 26, 27). $F_1$ carries out slow net ATP hydrolysis when a single molecule of ATP is bound and $10^6$-fold faster catalysis when ATP is saturating (2), a much more dramatic difference than is seen with Rho. For $F_1$, the results from binding fluorescence change and active/inactive subunit mixing approaches agree that accelerated catalysis (promotion) is maximal when all three active sites are occupied by ATP but there is disagreement concerning whether two sites are sufficient (28–31). The steady-state trapping experiment that was carried out here with Rho showing three sites filled during steady-state catalysis does not appear to have been performed with $F_1$.

Our ongoing efforts to understand the molecular details of directional travel by Rho along RNA and its role in transcription termination have exploited both pre-steady-state kinetics techniques and equilibrium binding studies. The results have led to the present model including ordered sequential catalysis among the three Rho active sites and the prediction that one or more enzyme conformation changes brought about by using the energy from ATP hydrolysis are likely to be rate-determining. The present results extend this model to the enzyme steady state. Catalytic cooperativity and a catalytic cycle in which the predominant enzyme form is saturated with ATP are both predicted by the model and confirmed by the data presented here.

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Fig. 4. Approximate relative duration of different parts of a Rho catalytic cycle. As determined from pre-steady-state experiments and by trapping from the steady state, ATP and RNA binding, the chemistry step, and product ADP and $P_i$ off-rates must all be fast, leaving RNA dissociation and enzyme conformation change(s) as the remaining candidates for the slow step(s) of catalysis. The question mark indicates that the position of this step in the cycle is uncertain.