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ATPase activity of transcription-termination factor rho: Functional dimer model

(rho protein/RNA-dependent ATPase/RNA cofactor binding)

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ABSTRACT **Transcription-termination factor rho of *Escherichia coli* functions as an RNA-dependent ATPase that causes transcript release at specific rho-dependent termination sites on the DNA template. Rho exists as a hexagon of identical subunits, physically organized as a trimer of dimers with D_3 symmetry. The structural asymmetry of the dimer is reflected in the binding properties of rho; each dimer has a strong and a weak binding site for both the ATP substrate and the RNA cofactor. Here we use homopolynucleotides in competition and complementation experiments to characterize the ATPase activation properties of the cofactor binding sites of the functional rho dimer. We show that (*i*) no ATPase activity is observed unless both the high- and the low-affinity cofactor binding sites of the functional rho dimer are occupied; (*ii*) saturating levels of poly(rC), poly(rC) in combination with poly(rU), or poly(rU) alone can fully activate the ATPase of rho; and (*iii*) poly(dC) can serve as a fully competitive inhibitor of half of the ATPase activity of rho when one of the cofactor sites is filled with poly(rC). These observations lead to a set of phenomenological rules that describe the cofactor dependence of the ATPase activation of the functional dimer of rho and help to define a mechanistic basis for interpreting rho function in termination.**

Rho protein is required to release nascent RNA transcripts from ternary (polymerase–RNA–DNA) transcription complexes that are paused at specific rho-dependent termination sites within the *Escherichia coli* genome (see refs. 1 and 2 for reviews). The termination activity of rho is dependent on the activation of an RNA-dependent ATPase with specific polynucleotide cofactor requirements. These specificities have been described in terms of two types of cofactor sites on the rho molecule (3, 4). In this paper we further define the properties of these ATPase activation sites.

Rho exists (and presumably functions) under physiological conditions as a hexamer of six identical subunits (5–8). Each subunit contains one ATP (substrate) and one RNA (cofactor) binding site (9). Recent binding studies have shown that the six ATP binding sites of the hexamer fall into two affinity classes of three sites each (10). It has also been shown that the six RNA binding sites of the rho hexamer can bind six RNA oligonucleotides and that these cofactor binding sites are also divisible into two classes of three sites each on the basis of binding affinity (ref. 11; also Y. Wang and P.H.v.H., unpublished results).

Subunit association studies (12) demonstrate that rho hexamer formation proceeds through a distinct and stable dimer intermediate. Geiselmann *et al.* (13) have demonstrated that rho exists as a hexagon with D_3 symmetry, meaning that the hexamer can be treated as a trimer of structurally asymmetric dimers. The subunits of each of these dimers are related by a C_2 symmetry axis. Each dimer has two types of subunit interaction surfaces as well as two substrate (ATP) and two cofactor (polynucleotide) binding sites. Here we use homopolynucleotide cofactors to build on this structural and binding site information to develop a functional dimer model of the activation of rho ATPase by RNA.

MATERIALS AND METHODS

Rho Protein. The transcription-termination factor rho used in these studies was purified from rho-overproducing *E. coli* strain AR120 containing plasmid p39-AS (14) as described (15). Concentrations of rho protein were determined spectrophotometrically by using a molar extinction coefficient ($\varepsilon_{M,280}$) of 1.49×10^4 $M^{-1} \cdot cm^{-1}$ (8). All protein concentrations are reported in units of rho monomers.

Polynucleotides and Biochemicals. All homopolynucleotides were purchased from P-L Laboratories or from Sigma. Aliquots of stock polynucleotide were 5′-end-labeled by kinase treatment with [γ-^{32}P]ATP and subjected to electrophoresis on polyacrylamide gels to estimate size distributions. No polynucleotide preparations with average chain length less than 200 nucleotide residues were used to avoid any cofactor-length dependence of the observed ATPase activity. Polynucleotide concentrations are reported in units of nucleotide residues. All other biochemicals were obtained from Sigma.

ATPase Assays. A coupled enzyme assay with spectrophotometric detection was used to determine steady-state rates of ATP hydrolysis at 37°C (16). The oxidation of NADH was monitored at 340 nm. The assay buffer contained 0.1 mM dithiothreitol, 0.1 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl, and 20 mM Hepes at pH 7.8. Each 1-ml reaction volume contained 8 units of phosphokinase, 8 units of lactic dehydrogenase, 1 mM phospho*enol*pyruvate, and 200 μM NADH. ATP concentrations were maintained at 50 μM in all assays. Each assay was initiated by adding an aliquot of rho to a preequilibrated mixture containing all of the other components. Measurement errors were less than ±5% of the reported ATPase rates.

RESULTS

Activation of rho ATPase Activity by Homopolynucleotide Cofactors. Poly(rC) activates the ATPase activity of rho in a sigmoidal fashion. A typical activation curve of rho ATPase as a function of poly(rC) added is shown in Fig. 1. As will be justified extensively below, we interpret the sigmoidal shape of this curve to indicate that (*i*) activation of rho ATPase requires the simultaneous occupancy of both polynucleotide cofactor sites of the functional dimer, (*ii*) the binding affinities of these sites for poly(rC) are different, and (*iii*) the two

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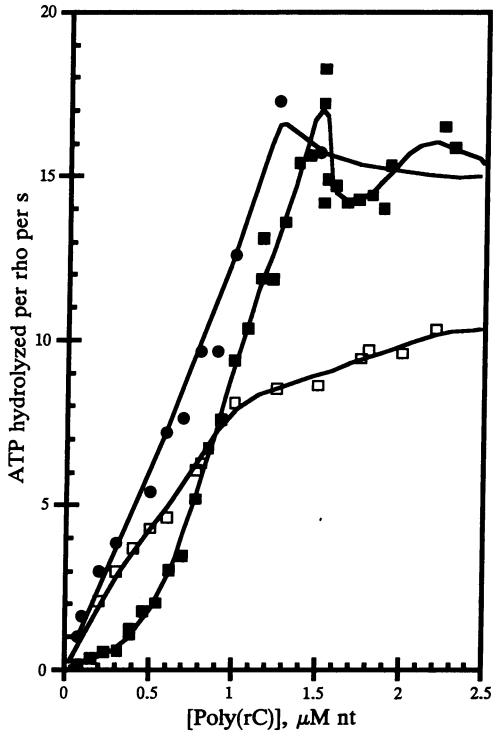

FIG. 1. Activation and reverse complementation of rho ATPase activity. Steady-state ATPase rates induced by the addition of the indicated concentrations of poly(rC) to 50 nM solutions of rho monomer alone (■), in the presence of 20 μM poly(rU) (●), or in the presence of 5 μM poly(dC) (□). nt, Nucleotide.

sites within individual functional dimers are filled noncooperatively. We note that this interpretation is consistent with the observation (Fig. 1) that the total ATPase activity of rho was less than half-maximal at half-saturating concentrations of poly(rC). Thus, if the cofactor sites within individual dimers are indeed filled noncooperatively, a simple Poisson distribution calculation [weighted for the different affinities of poly(rC) for the two cofactor binding sites of the functional dimer] shows that considerably less than half of the functional dimers of rho will have both cofactor sites occupied at half-saturating poly(rC) concentrations.

Activation of the rho ATPase required 130 to 160 or more residues of poly(rC) per rho hexamer (see peak of poly(rC) activation profile in Fig. 1). The exact magnitude of this apparent activation-site size may depend on experimental conditions but always exceeds the binding-site size of ≈70 nucleotide residues per rho hexamer previously defined (5, 17, 18). A structural interpretation of this functional-site size will be presented elsewhere.

Polynucleotides other than poly(rC) can also interact with the cofactor binding sites of the functional dimer of rho. Richardson (3) showed that poly(rU) can activate rho ATPase, though much less effectively than poly(rC), and McSwiggen *et al.* (18) demonstrated that poly(rU) binds to rho about 20-fold more weakly than poly(rC) at the salt concentration of our assays. Full ATPase activation of rho by poly(rU) required polynucleotide concentrations that greatly exceed the 20-fold difference in binding affinity of poly(rU) and poly(rC) (data not shown). Poly(rC) and poly(dC) bind rho competitively and with equal affinity (18). However, poly(dC) alone cannot activate rho ATPase. We show here that binding of poly(dC) to one of the cofactor sites of the functional dimer resulted in full competitive inhibition of half of the poly(rC)-activated ATPase of rho.

The experiments presented in this paper show that cofactor activation of the ATPase activity of rho requires simultaneous occupancy of both polynucleotide binding sites of the functional dimer and that the magnitude of the ATPase activation achieved depends on the base and the sugar compositions of the polynucleotide(s) bound. A set of phenomenological rules that describe the activation of rho ATPase by polynucleotide binding is developed below and is summarized in the *Discussion* (see Fig. 4).

Competition and Complementation of Poly(rC) Activation of rho ATPase by Other Homopolynucleotide Cofactors. Steady-state ATPase competition and complementation studies with homopolynucleotides can be used to differentiate and characterize the two cofactor binding sites of the functional rho dimer. Poly(rU) was added to assays containing poly(rC) to determine whether this cofactor complements or competes with poly(rC) in the activation of rho ATPase. Fig. 1 shows an activation curve for a 50 nM solution of rho as a function of poly(rC) concentration in the presence of 20 μM poly(rU). As these complementation results show, the characteristic sigmoidal shape of the poly(rC) activation curve was abolished by the addition of this amount of poly(rU), although the concentration of poly(rC) required to fully activate the rho ATPase was only marginally less than was required in the absence of poly(rU). We interpret these results as follows.

Poly(rU), which has an ≈20-fold lower overall binding affinity for rho than poly(rC) (18), can saturate one of the two cofactor binding sites of the functional dimer under the protein and polynucleotide concentration conditions of this experiment. We refer to this site as the high-affinity site of the functional dimer, or "Site 1." On the other hand, we will show (see Fig. 2) that poly(rU) is unable to bind to the other (low affinity) site ("Site 2") of the functional dimer at this (relatively low) poly(rU) concentration. We do know that very high concentrations of poly(rU) alone can fully activate rho ATPase (18). Thus, in terms of the present model, Site 2 as well as Site 1 binding can be saturated by poly(rU) at very high concentrations, thereby fully activating the rho ATPase.

Poly(rC) can effectively occupy Site 2 and stimulate full ATPase activity without sigmoidicity by complementing the poly(rU) that is prebound in Site 1 at the poly(rU) concentrations of Fig. 1. Therefore, the activation curve of rho ATPase by poly(rC) in the presence of sufficient poly(rU) to saturate Site 1 is initially hyperbolic. We observed that this activation curve starts at a zero ATPase rate (Fig. 1), indicating that functional dimers with cofactor [here poly(rU)] bound only in Site 1 are not activated. In keeping with this interpretation, Fig. 1 shows that the amount of poly(rC) required for maximal stimulation of ATPase activity in the poly(rU)-complementation experiment was only slightly less in the presence of this amount of poly(rU) than in its absence. This follows because, both in the poly(rC) alone and in the poly(rU) complementation experiments, it is the saturation by poly(rC) of the weaker-binding Site 2 (to which poly(rU) does not bind at these concentrations) that drives rho ATPase activity to its maximal rate.

The above interpretation of these data is confirmed in Fig. 2, where rho ATPase activity is plotted as a function of poly(rU) added to rho solutions containing various subsaturating levels of poly(rC). A saturating amount of polynucleotide is defined as the number of moles of poly(rC) residues needed to just reach maximal ATPase activation (here ≈160 residues per rho hexamer; see Fig. 1). Insignificant changes in ATPase activity were observed when poly(rU) was added to rho solutions containing saturating concentrations of poly(rC) (Fig. 2, top curve). This is consistent with the above interpretation—i.e., that although poly(rU) competes poorly with poly(rC) for the cofactor binding sites of rho, it nevertheless activates rho ATPase in the same fashion as poly(rC) and therefore should have little additional effect on the observed ATPase rate under these conditions of full poly(rC) saturation.

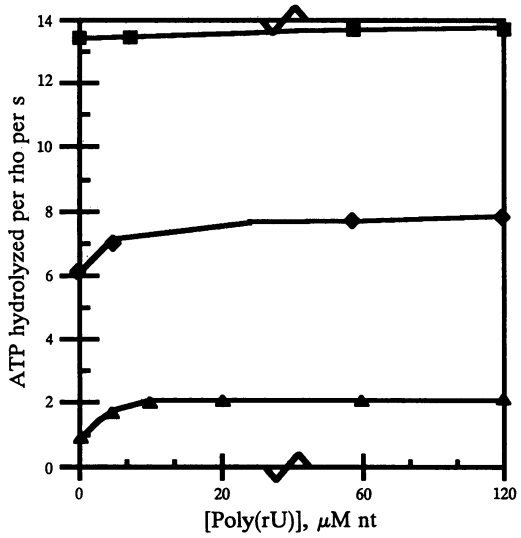

FIG. 2. Competition and complementation of poly(rC)-induced rho ATPase activity by poly(rU). Steady-state ATPase rates induced by the addition of the indicated concentrations of poly(rU) to 50 nM solutions of rho monomers containing saturating (■); half-saturating (◆); and quarter-saturating (▲) concentrations of poly(rC). A saturating concentration of poly(rC) is defined at this concentration of rho as ≈1.5 μM poly(rC) (see Fig. 1). nt, Nucleotide.

Additional insight is derived from the lower curves of Fig. 2, which show poly(rU) complementation of the ATPase activity of rho solutions that are half- or quarter-saturated with poly(rC) in terms of the functional ATPase-site size. The observed ATPase rate of these rho solutions increased significantly with the initial addition of poly(rU), and then effectively leveled out at rates that were approximately one-half or one-quarter of those achieved with saturating levels of poly(rC). This complementation reflects the initial distribution of poly(rC) between Sites 1 and 2, as discussed above in connection with the observed sigmoidal poly(rC) activation curve of Fig. 1.

In Fig. 2 the poly(rU) added in the initial phases of the titration should fill any high-affinity cofactor sites that may not have been occupied by poly(rC). Poly(rU) competes with the poly(rC) for the high-affinity sites, making any poly(rC) that is displaced by the added poly(rU) available to complete binding to unfilled low-affinity sites within the rho dimers. Maximal ATPase rates were achieved, since functional dimers could be activated by the presence of either poly(rC) or poly(rU) in Site 1. The gradual further increase seen in the overall ATPase rate with the addition of very large concentrations of poly(rU) must reflect the very weak binding of poly(rU) to the remaining unfilled low-affinity sites of the functional dimer. This experiment also supports the view that both cofactor sites of a functional dimer must be occupied to achieve any activation of rho ATPase.

Poly(dC) can bind as a competitive inhibitor to one of the two sites of the functional rho dimer, and yet, by occupying this site (Site 1), can permit the activation of the other rho subunit of the dimer by poly(rC). The top curve of Fig. 3 *Left* illustrates this effect on the overall rho ATPase rate when increasing amounts of poly(dC) were added to an initially poly(rC)-saturated rho solution. As this result shows, increasing concentrations of poly(dC) clearly drive the ATPase rate to half of that obtained with saturating poly(rC).

The inhibition by poly(dC) of half the maximal poly(rC)-stimulated rho ATPase is fully competitive, as shown by the linear Eadie–Hofstee plot for this fraction of the poly(rC)-activated rho ATPase (Fig. 3 *Right*). A mixed, partial, or noncompetitive mode of competition would result in nonlinear Eadie–Hofstee plots. The measured inhibition constant (K_i) for poly(dC) is $6 \times 10^8\ M^{-1}$, which is only slightly less than the binding constant of $1 \times 10^9\ M^{-1}$ previously measured for poly(dC) (18). We consider that this K_i value represents the apparent inhibition constant for cofactor binding to Site 1 (the high-affinity site) of the rho dimer.

Poly(dC) acts as a classical competitive inhibitor of an independent catalytic site (Fig. 3 *Right*), and the fully poly(dC)-inhibited functional dimer catalyzes ATP hydrolysis at half of the maximal rate. These facts suggest that each fully liganded rho subunit (i.e., containing an ATP substrate and an RNA cofactor) hydrolyzes ATP at a rate that is independent of the nature of the cofactor bound to the other monomer of the functional dimer, provided that a polynucleotide cofactor (or inhibitor) is indeed bound in the other site and that the substrate and cofactor concentrations available exceed the K_m values for each site. We note also that the catalytic competition results obtained at saturating poly(rC) concentrations are consistent with the earlier direct binding measurements (18) that showed that poly(rC) and poly(dC) have similar binding affinities for rho. It appears that these previously measured binding affinities may have reflected competitive binding at Site 1 only (see *Discussion*). The enzymatic results presented here show that these polynucleotide cofactors must bind and interact differently with Site 2.

It is important to state explicitly that the ratio of nucleotide residues bound to rho sites present is an important variable in these experiments. The poly(rC) activation curve of Fig. 1 shows the ratio of poly(rC) nucleotides to rho to be a critical parameter that determined the level of ATPase activation. Small variations in concentration yielded significant variations in the observed steady-state ATPase, in the presence and absence of competing or complementing polymers. The maximal poly(rC)-stimulated ATPase activity of rho occurred over a very small range of relative concentrations; slight variations in the ratio of rho to poly(rC) nucleotide concentrations resulted in decreased activity. In addition, ≈1.5 μM poly(rC) could maximally stimulate the ATPase activity of 50 nM rho, yet about the same concentration of poly(rC) was also needed to yield maximal reverse complementation activity when saturating concentrations (≈1.6 μM) of poly(dC) were used. This result suggests that both polynucleotides can coexist on the same rho hexamer and exhibit their maximal effects. The order of addition of various cofactor components and their stoichiometries relative to rho concentrations can perturb both the steady-state and single-turnover ATPase kinetics of rho. Further details will be presented elsewhere.

Fig. 3 *Left* shows that the addition of low concentrations of poly(dC) to rho-containing solutions that were less than fully saturated with poly(rC) induced an initial increase in the observed ATPase activity in a fashion qualitatively analogous to the poly(rU) addition effects of Fig. 2. Again, we suggest that this complementation reflects a redistribution of the available poly(rC) into the low-affinity sites of the functional dimers, while the added poly(dC) binds to the high-affinity sites. We note, however, that these effects are a more abrupt function of polynucleotide concentration when poly(dC), rather than poly(rU), served as the competing cofactor for Site 1. Fig. 3 *Left* shows that higher concentrations of poly(dC) eventually competitively blocked all of the poly(rC) from Site 1, yielding a final V_{max} for rho ATPase of half the value obtained with saturating poly(rC) alone. Experiments involving poly(dC) titration of rho solutions containing quarter-saturating amounts of poly(rC) (Fig. 3 *Left*) also confirmed that the observed ATPase rate is ultimately limited by the amount of poly(rC) that is available for binding to Site 2.

We observed less than one-quarter of the maximal enzymatic rate when low concentrations of added polynucleotide were added (Fig. 3 *Left*), since statistically less than one-

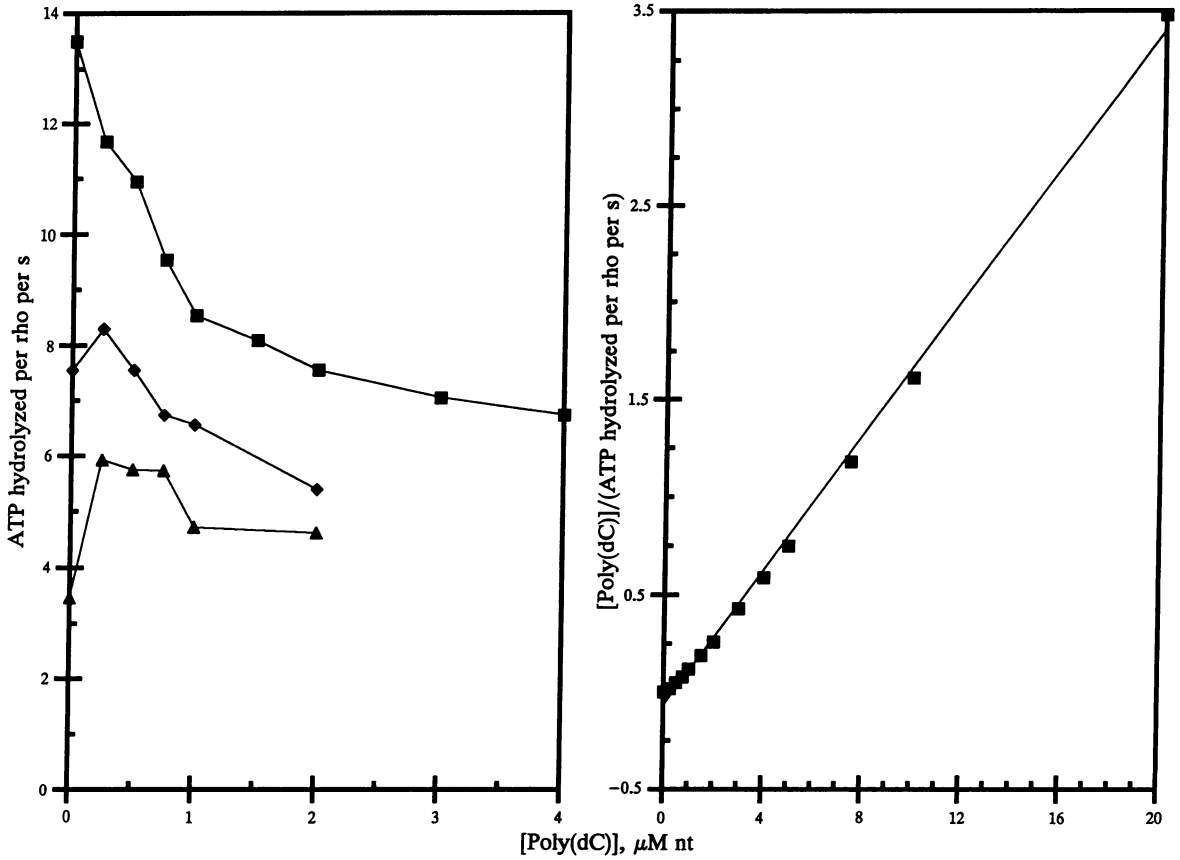

FIG. 3. Complementation and competition of poly(rC)-induced rho ATPase activity by poly(dC). (*Left*) Steady-state ATPase rates of rho induced by the addition of the indicated concentrations of poly(dC) to 50 nM solutions of rho monomers. ■, Saturating poly(rC); ◆, half-saturating poly(rC); ▲, quarter-saturating poly(rC). (*Right*) An Eadie–Hofstee plot of the saturating poly(rC) data (top curve) of *Left*; saturating poly(rC) is defined as in the legend to Fig. 2. nt, Nucleotide.

quarter of the functional dimers has both cofactor sites occupied. Excess poly(dC) could only drive the final velocity up to one-quarter of the maximal ATPase rate because of the limited amount of poly(rC) available to bind to the unoccupied low-affinity sites. The model that emerges from these observations predicts that a functional dimer carrying poly(rC) in both cofactor sites will catalyze ATP hydrolysis at the maximal rate, while dimers carrying poly(dC) in Site 1 and poly(rC) in Site 2 will hydrolyze ATP at $V_{max}/2$. There was no further change in the apparent ATPase activity of rho with further addition of poly(dC), in contrast to the gradual increase in ATPase rates seen in Fig. 2 with further addition of poly(rU).

The above interpretations are consistent with the results of the steady-state reverse complementation assays of Fig. 1, in which Site 1 of the functional rho dimer was filled with poly(rU) [or poly(dC)] in addition to the added poly(rC). No ATPase activity was observed with either of these polynucleotides without the addition of poly(rC), as expected if both cofactor sites must be occupied to activate rho ATPase. The poly(rU)-containing samples yielded activation curves that are initially hyperbolic and that saturate at almost the same poly(rC) concentration as does the activation curve for poly(rC) alone. In the complementation experiment, poly(rC) will eventually competitively block poly(rU) from Site 1 in addition to filling Site 2, but the functional dimers that contain saturating poly(rU) in Site 1 and poly(rC) in Site 2 will catalyze ATP hydrolysis at essentially the same rate as those that carry poly(rC) in both cofactor binding sites.

The reverse complementation curve of Fig. 1, obtained when poly(rC) was included in a poly(dC)-containing solution of rho, is more complex. This curve reflects both complementation as poly(rC) filled Site 2 and then competition as this ribopolynucleotide blocked poly(dC) from Site 1. We note that this curve is also initially hyperbolic rather than sigmoid; thus here poly(dC), which at high poly(rC) concentrations is an inhibitor (Fig. 3 *Left*), also binds first to Site 1 and therefore [like poly(rU)] acts as an initial activator of poly(rC)-dependent ATPase. Eventually both sites were filled with poly(rC), resulting in maximal ATPase activation. However, because of the high affinity of poly(dC) for Site 1, full activation was not achieved at the levels of poly(rC) added in the poly(dC) reverse complementation experiments shown in Fig. 1.

An Eadie–Hofstee plot (or equivalent) of the rate of poly(rC)-stimulated rho ATPase in the presence of saturating poly(dC) yields an apparent K_m for the poly(rC) cofactor of $\approx 8 \times 10^5\ M^{-1}$, which is significantly less than the binding constant measured for either of these polynucleotides by McSwiggen *et al.* (18). The above interpretation suggests that this K_m reflects the cofactor activation properties of the low-affinity site of the functional rho dimer (Site 2).

DISCUSSION

In earlier work we and others have shown that rho is structurally and functionally a trimer of dimers under physiological conditions (6, 8, 9, 12, 15). The substrate and cofactor binding sites of the individual rho subunits within each dimer differ in their affinity for ATP and for RNA (refs. 10 and 11; also Y. Wang and P.H.v.H., unpublished results). In this paper we have presented steady-state ATPase cofactor complementation and competition data that permit us to characterize the cofactor sites of the functional rho dimer in

terms of polynucleotide interaction specificity. Our findings are summarized schematically in Fig. 4, which shows the levels of ATPase activation that are obtained as each of the cofactor sites of the functional dimer is saturated with the indicated polynucleotide. Site 1 in Fig. 4 represents the high-affinity site and Site 2 represents the low-affinity site, as defined above.

Our conclusions are summarized in Fig. 4. We find that (*i*) no ATPase activity is observed without bound cofactor; (*ii*) no ATPase activity is observed if either cofactor site is unoccupied; (*iii*) full ATPase activity is observed if both sites are saturated with either poly(rC) or poly(rU) or with complementing mixtures of both cofactors [though poly(rU) binds to both sites much more weakly than does poly(rC), and in practice Site 2 can only be effectively saturated with poly(rC)]; and (*iv*) poly(dC) is a fully competitive inhibitor of poly(rC)-stimulated rho ATPase at Site 1, but filling Site 1 with poly(dC) complements the activation of Site 2 by poly(rC) and results in a maximal ATPase rate of $V_{max}/2$. We conclude that the basic catalytic unit of rho is a dimer, that the two types of polynucleotide cofactor activation sites occur in pairs on these dimers within the rho hexamer, and that the effective interactions of the two cofactor sites of the functional dimer with polynucleotides, as reflected in ATPase activation, are quite different.

How do these ATPase activation results relate to previous binding studies? McSwiggen *et al.* (18) showed that poly(rC) and poly(dC) bind competitively and with equal affinity to rho

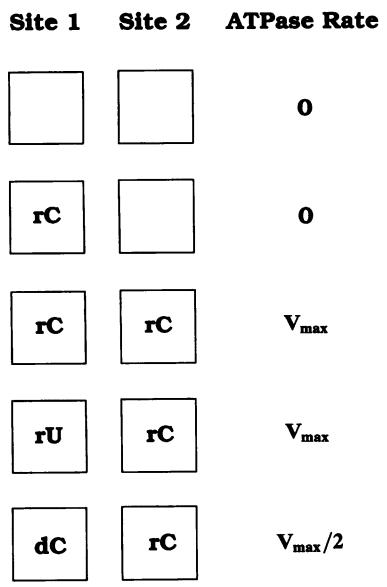

FIG. 4. Functional dimer model of transcription-termination factor rho. Each horizontal set of two boxes represents the two cofactor sites of the functional dimer of rho. Several states of occupancy are illustrated; empty boxes are unoccupied, while filled boxes are labeled with the polynucleotide that occupies that site. The relative steady-state ATPase rates that result from the indicated occupancies are shown beside each horizontal set.

hexamers with a binding constant of $\approx 10^9\ M^{-1}$ (per hexamer), while poly(rU) binds ≈20-fold more weakly. The absolute and relative values of these binding affinities are essentially the same as the cofactor K_m values that have been measured for the interaction of these moieties with Site 1 of the functional rho dimer in this study. We suggest, for steric and connectivity reasons, that long polynucleotides bound to rho must wrap through all six RNA cofactor binding sites of the hexamer, but that the effective binding affinity for such polynucleotides reflects primarily their interactions with the high-affinity sites (Sites 1), and that the interactions of the polynucleotide chain in passing through the low-affinity sites (Sites 2) contribute little or nothing to the total free energy of binding. Recent binding studies of oligonucleotides to rho (Y. Wang and P.H.v.H., unpublished results) are fully consistent with this view.

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