A physical model for the translocation and helicase activities of *Escherichia coli* transcription termination protein Rho

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Contributed by Peter H. von Hippel, April 20, 1993

ABSTRACT Transcription termination protein Rho of *Escherichia coli* interacts with newly synthesized RNA chains and brings about their release from elongation complexes paused at specific Rho-dependent termination sites. Rho is thought to accomplish this by binding to a specific Rho “loading site” on the nascent RNA and then translocating preferentially along the transcript in a 5′ → 3′ direction. On reaching the elongation complex, Rho releases the nascent RNA by a 5′ → 3′ RNA-DNA helicase activity. These translocation and helicase activities are driven by the RNA-dependent ATPase activity of Rho. In this paper we propose a mechanism for these processes that is based on the structure and properties of the Rho protein. Rho is a hexamer of identical subunits that are arranged as a trimer of asymmetric dimers with D₃ symmetry. The binding of ATP and RNA to Rho also reflects this pattern; the Rho hexamer carries three strong and three weak binding sites for each of these entities. The asymmetric dimers of Rho correspond to functional dimers that can undergo conformational transitions driven by ATP hydrolysis. We propose that the quaternary structure of Rho coordinates the ATP-driven RNA binding and release processes to produce a biased random walk of the Rho hexamer along the RNA, followed by RNA-DNA helicase activity and transcript release. The proposed model may have implications for other hexameric DNA-DNA, RNA-DNA, and RNA-RNA helicases that function in replication and transcription.

Transcript termination occurs at intrinsic and Rho-dependent termination sites along the *Escherichia coli* genome. RNA polymerase alone can bring about termination at intrinsic sites, but Rho protein is required to release the nascent transcript at Rho-dependent sites. Termination at both types of sites requires a substantial and highly localized (in terms of template position) thermodynamic destabilization of the transcription complex that puts the complex into a “termination mode” from which the nascent RNA can be released (1). At intrinsic termination sites, this process may involve the destabilization of the upstream portion of the DNA-RNA hybrid within the transcription bubble by the competitive formation of an RNA hairpin within the nascent transcript, coupled with the presence of a particularly unstable rU-dA sequence within the downstream portion of the hybrid (2–4). The ATP-dependent RNA-DNA helicase activity of Rho (5, 6) is thought to bring about equivalent thermodynamic destabilization of the hybrid at Rho-dependent termination sites. Termination efficiency at both types of sites may be modulated by additional protein factors.

The function of Rho protein in transcript termination (for reviews, see refs. 3, 7, and 8) involves three processes. The protein first binds to the nascent RNA transcript at a “loading site” that is ≈70 nt long, contains at least 10% cytosine residues, and is largely unencumbered with secondary structure (9–12). This binding activates the RNA-dependent ATPase activity of Rho (13) and results in a preferential 5′ →

3′ movement of the protein along the nascent RNA to elongation complexes paused at specific Rho-dependent termination sites on the DNA template. Rho then engages its ATPase-dependent RNA-DNA helicase activity to release the RNA from the transcription complex (5). The sequence specificity of Rho-dependent termination sites appears to reflect the extended dwell-time of the elongation complex at these positions (14–16). This extensive pausing allows Rho proteins that are translocating along the nascent RNA to “catch up” with the transcription complex at these sites, with the resultant termination efficiency depending on the “kinetic coupling” of the rate of movement of Rho along the nascent transcript and of RNA polymerase along the DNA template (1, 17, 18).

The development of this view of Rho function has been paralleled by progress in elucidating its structural and enzymatic properties. It has been shown that Rho exists under physiological conditions as a hexamer of identical subunits (19–23), organized as a trimer of asymmetric dimers with overall D₃ symmetry (24). This symmetry is reflected functionally in the presence of three strong and three weak ATP (substrate) and three strong and three weak RNA (cofactor) binding sites per Rho hexamer (25–28). The interaction of Rho with RNA is critical to all the activities of the protein. Thus RNA binding is required to activate the RNA-dependent ATPase activity of Rho (29, 30), which drives the protein conformational changes that are required for ordered RNA release from the subunits of the Rho hexamer (see below). The ATP-dependent 5′ → 3′ RNA-DNA helicase action of Rho can be demonstrated in the absence of polymerase with a construct consisting of a single-stranded piece of RNA carrying a Rho loading site that is hybridized to DNA at the 3′ end (5).

In this paper we combine these data to suggest a physical model for the function of Rho in transcript termination. Elsewhere this model is extended (refs. 28, 31, and 32 and unpublished results) to couple the translocation and helicase activities of Rho to the details of the ATPase and RNA binding and release cycles that underlie its function.

Structural and Enzymic Properties of Rho and Rho-RNA Complexes

Each Rho subunit contains three functional domains. The RNA binding site has been localized to the N-terminal portion of the protein (33–36), the ATP binding site appears to be located in the central portion of the primary sequence (37, 38), and subunit interaction sites have been proposed in the C-terminal region (35). Rho monomers associate to form a hexamer with D₃ symmetry under physiological conditions.

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Six molecules of ATP can bind to the Rho hexamer; three bind with high affinity (25) and three bind 20- to 30-fold more weakly (26). Thus ATP binding creates (or induces) an asymmetry within the Rho hexamer and the smallest repeating unit of Rho can be considered to be a dimer of nonequivalent subunits in which one of the monomers allows tight binding of ATP and the other is in a low-ATP-affinity state. Interactions between subunits couple conformational changes within the functional dimer (31).

RNA binding studies with short oligonucleotide cofactors (27, 32) have been used to probe the details of the cofactor binding interactions of Rho. Here also three short (<20 nt) RNA cofactors bind tightly per Rho hexamer (27) and three bind with lower affinity (32). The binding of oligonucleotide cofactors to the three high-affinity sites induces a change in RNA conformation, as detected by circular dichroism, whereas binding to the low-affinity sites has little or no effect on the CD signal. Studies with fluorescent RNA analogues have also revealed differences between the two classes of RNA sites (39). Thus RNA binding induces further asymmetry at the dimer level.

Titration of Rho with polymeric RNA shows that RNA chains bind to Rho with high affinity and with an overall binding site size of 70 nt per Rho hexamer, corresponding to 11–12 nt per Rho subunit (40). Furthermore, Rho binding protects the entire “hexamer-site size” of RNA against digestion by RNase A (21), suggesting that the RNA chain is tightly bound to the hexamer over most of its length. These facts suggest not only that the RNA chain binds to the strong RNA binding sites on alternate subunits but also that the RNA binds to (or at least passes through) the intervening weak binding sites of the hexamer. Thus we suggest that these latter sites lie on the natural “connectivity path” of the RNA in going from one strong RNA binding site to the next and that binding to these sites is favored by the high local concentration of RNA segments on the surface of the hexamer. To facilitate model representations and to emphasize these differences in binding affinity (see figures and text below), we show Rho subunits with strong RNA binding sites oriented with “correct” polarity relative to the RNA chain and subunits with weak-affinity sites oriented with “incorrect” chain polarity.

Binding studies of short RNA cofactors to Rho (27, 32) have shown that the affinity for such ligands (at least for the three strong sites) increases substantially with oligomer length up to 9 nt (27, 32, 41). Since the average RNA site size per Rho subunit is 11–12 nt, this suggests that 9 nt interact favorably with the RNA sites, leaving only 2 or 3 nt to span the distance between cofactor binding sites within the hexamer.

A Physical Model for Rho Function

Path of the RNA Chain. There are several possible arrangements of the RNA binding sites within the Rho dimer that are consistent with D_3 symmetry. Two extreme arrangements are shown in Fig. 1. In Fig. 1A, the polar binding sites are oriented so that they point toward the twofold axis, whereas in Fig. 1B, the binding sites are aligned parallel to the dimer interface. The path of an RNA chain binding to the two arrangements is also shown. The important difference between these arrangements is that in Fig. 1B the RNA binds to each subunit site of the dimer with the same polarity, whereas in Fig. 1A the polynucleotide chain binds to alternate sites with opposite polarity.

We note that the distance between binding sites on the dimer could be close to zero in Fig. 1A, whereas the minimal length of the spacer RNA in Fig. 1B would have to be much greater to be compatible with a D_3 arrangement. Neutron scattering experiments have shown that the RNA is probably

FIG. 1. (A and B) Two possible paths for RNA in passing through the RNA binding sites of an asymmetric dimer of Rho. The different conformational states of the Rho subunits (see text) are symbolized by circles and (rounded) squares. The polar RNA binding site on each Rho subunit is represented by an open arrow. The polarity of the RNA chain is represented by arrows pointing in the 5' → 3' direction. The twofold axis of symmetry is perpendicular to the plane of the paper and lies at the midpoint between the two Rho monomers.

wrapped around the periphery of the Rho hexamer (23). The distance between the centers of mass of adjacent Rho monomers within a hexamer is >40 Å (23); therefore, the average distance between the centers of the RNA binding sites on two adjacent subunits within the hexamer must also be >40 Å. Since only 2 or 3 nt per subunit are available to span the intersite distance between adjacent RNA binding sites, this geometrical constraint eliminates arrangements of the type shown in Fig. 1B and leaves us with arrangements related to Fig. 1A to represent the path of the RNA through the dimer units of the Rho hexamer.

At the hexamer level we can also consider two extreme models, both of which are consistent with the relative arrangement of cofactor sites within the dimer represented in Fig. 1A. In the representation shown in Fig. 2A, the RNA chain enters and exits the overall hexamer parallel to the circumference of the hexamer. In Fig. 2B, the chain enters and exits perpendicular to the circumference. Symmetry considerations favor the latter arrangement, in that the hexamer in Fig. 2B (but not the hexamer in Fig. 2A) has exactly one interdimer interface connecting subunits of different conformation (circle to square). During a catalytic cycle this interface is always located between the dimers through which the RNA enters or exits and could thus “activate” these dimers for the next step of the helicase cycle (see below). To permit specificity in what follows, we develop our physical model of Rho function by using an RNA path through the hexamer such as that shown in Fig. 2B, which is then converted to the more schematic representation of Fig. 3A to facilitate visualization of subsequent mechanistic processes.

Asymmetry of the RNA-Complexed Rho Hexamer. The unliganded Rho hexamer has D_3 symmetry. The binding of either the ATP substrate or the RNA cofactor (or both) reduces this symmetry to pseudo- D_3 because the subunits within each Rho dimer are now different (of course this asymmetry could also preexist in the unliganded hexamer). This level of conformational asymmetry is represented in Fig. 3 by showing each dimer with a circle subunit and a square subunit. Additional asymmetry is created by binding the RNA chain to the hexamer. Thus the middle dimer in Fig. 3A now differs from the other two in that neither end of the RNA emerging from this dimer is free; i.e., both ends are bound to flanking dimers. The dimer at which the RNA enters the hexamer differs from that through which the RNA exits

FIG. 2. Schematic drawings of the D_3 -symmetric Rho hexamer bound to an RNA chain. Components are represented as in Fig. 1. The parts of the RNA chain and the RNA binding sites that are hidden behind portions of the Rho hexamer are shown dotted. One surface of each Rho monomer is shaded to indicate that adjacent subunits expose opposing "faces" at the "top" and "bottom" surfaces of the hexamer. (A) The RNA binding sites of the Rho subunits are shown parallel to the overall plane of the hexamer. (B) The RNA binding sites point toward the twofold axis of each dimer at one end and toward the center of the hexamer at the other. As a result, the RNA passes from the bottom to the top surface of the hexamer at each dimer. The RNA chain as a whole enters at the bottom surface of the hexamer and exits at the top surface.

because in the former the free end of the RNA is held by a Rho subunit with "correct" RNA binding polarity, and in the latter the free end is held by a subunit with "incorrect" polarity. The binding of long RNA thus makes the Rho hexamer completely asymmetric, meaning that no Rho subunit within the hexamer is identical to any other. This asymmetry is utilized in formulating a model for directional movement of the hexamer along the RNA.

A Mechanism for Directional Translocation of the Hexamer Along the Nascent RNA Chain. We begin with the RNA chain bound to the hexamer in what we take as the minimum free energy configuration of the complex. This configuration is represented schematically in Fig. 3A. Here, as indicated above, the RNA chain binds to one of the subunits of each dimer (the circle) with correct polarity and to the other subunit (the square) with incorrect polarity. The affinity of the RNA for the dimer is essentially determined by the interaction with the stronger RNA binding site, which we take to be that located on the circle subunit. The ATPase cycle is initiated at the dimer level by the hydrolysis of the tightly bound ATP molecule. We propose that this hydrolysis is coupled with a conformational "switch" of the subunits within the dimer (circle becomes square and vice versa). The conformational change of the circle subunit into the square form weakens the strong RNA binding site on this subunit and thus decreases the overall affinity of the RNA for this dimer. The corresponding RNA segment dissociates from the dimer. The transition from Fig. 3A to Fig. 3B shows this reaction for the left dimer.

The two free ends of the RNA chain that are still attached to the hexamer in Fig. 3B will compete for binding to the free

FIG. 3. Schematic representation of the conformational and binding transitions of the Rho hexamer in the course of a reaction cycle of translocation along the RNA chain in the $5' \rightarrow 3'$ ($A \rightarrow B \rightarrow C$) or $3' \rightarrow 5'$ ($A \rightarrow D \rightarrow E$) direction. Rotation of each dimer of the Rho hexamer of Fig. 2 about its twofold axis results in the representation of the hexamer shown here. Topological and symmetry properties are fully conserved in this representation. Specific points on the RNA molecule are indicated by open and solid circles. (A) The Rho-RNA complex in the lowest free energy configuration. (B) The left dimer has undergone a conformational inversion and RNA release as a consequence of ATP hydrolysis. (C) The free RNA segment marked by the solid circle has bound to the free (left) dimer of B. (D) The right dimer has undergone conformational inversion and RNA release as a consequence of ATP hydrolysis. (E) The free RNA segment marked with the open circle has bound to the free (right) dimer of D.

dimer. This competition is more likely to be "won" by the free RNA segment located on the upper right in Fig. 3B than by the newly released segment, since the binding of the former segment will restore the original relationship between subunits and chain direction and return the Rho-RNA complex to its most stable configuration (i.e., with correct polarity of RNA binding to the circle subunit). The resulting configuration of the hexamer is shown in Fig. 3C. This hexamer is in all respects identical to the one in Fig. 3A, except that the hexamer has translocated along the RNA by one "dimer length" (22–24 nt) toward the 3' end of the RNA chain.

Exactly the same final result is attained if an RNA segment is released from the right-hand dimer, as shown in the transition from Fig. 3A to Fig. 3D, except that here the preferred rebinding of the RNA chain results in translocation of the hexamer by one dimer-length toward the 5' end of the RNA. Barring any specific effects of RNA sequence that may apply, the overall configurations shown in Fig. 3C and E are identical to one another and to that of the initial (stable) complex shown in Fig. 3A. The identity (except for position along the RNA chain) of Fig. 3A, C, and E shows that there is no thermodynamic bias that favors a translocation of the Rho hexamer in one direction over the other.

Clearly, a preferred direction of translocation must depend on kinetic factors, specifically on the relative probabilities of

hydrolyzing ATP and releasing RNA from the three dimers of Fig. 3A. This requirement is certainly structurally justified since, as described above, all three dimers are different. A concrete example can be used to illustrate such a kinetic mechanism. We have suggested above that RNA release is more likely from one of the outside dimers than from the middle dimer because the RNA segment bound to the middle dimer is held at both ends. We have also pointed out that the outside dimers differ structurally from one another. ATP hydrolysis and the resulting release of RNA requires that both subunits of a given dimer be initially occupied with RNA (ref. 31; Y.W., unpublished results). Thus ATP hydrolysis, and the subsequent release of the RNA segment, might be faster from the left dimer of Fig. 3A if that hydrolysis depends on the occupancy by RNA of the (weak) binding site on the square subunit. Occupancy of the square subunit of the left dimer of Fig. 3A would be higher than for the square subunit of the right dimer since the RNA segment on the left dimer is held in place by two adjacent strong binding sites.

RNA segment release could also be the kinetic step that distinguishes the left from the right dimer; this notion is supported by the demonstration that RNA release within an individual subunit RNA binding site appears to be polar and may be initiated at the 5' end of the bound RNA (32). An interchange of subunit conformations within the dimer after ATP hydrolysis would weaken the strong RNA binding interactions at the sites where the orientation of the RNA is parallel to that of the RNA binding site. This strong interaction is at the free end of the chain on the left, while the equivalent conformational change releases an "interior" chain segment on the right. These differences could lead to different net rates of RNA release from the two outside dimers. Clearly, RNA segments can also rebind (albeit more weakly) to the sites from which they have just been released. Such rebinding events would reduce the rate of hexamer translocation relative to the rate of ATP hydrolysis.

Regardless of the molecular details that underlie these differing reaction probabilities, the net effect will be a biased random walk of the Rho hexamer along the RNA chain in a 5' → 3' direction. The driving force for this reaction is clearly ATP binding and hydrolysis, which leads to subunit conformation changes within the dimer, preferential release of the free 5' end of the RNA segment, and preferential rebinding of the free 3' end of the chain. The chemical and enzymatic details of the coupling of these cycles will be considered elsewhere, but it is worth noting here that such models provide an explanation for the long-standing conundrum that the ATPase activity of Rho that is "turned on" by poly(rC) binding reaches a maximum value only when approximately twice the amount of poly(rC) needed to saturate the binding interaction has been added (31, 39, 42). Since here the catalytic cycle consists of the release of an RNA chain segment followed by the preferential rebinding of another (initially free) RNA segment, the overall ATPase activity of Rho could well be reduced when no "extra" RNA is available for rebinding with the correct polarity.

A Mechanism for Rho Helicase. A conceptual advantage of this translocation model is that the 5' → 3' RNA-DNA helicase activity of Rho can, in principle, be explained on the same basis. As the Rho hexamer moves toward the 3' end of the nascent RNA chain, it will encounter the double-stranded RNA-DNA hybrid of the paused elongation complex. We propose that Rho then continues to search for additional contiguous RNA of the correct orientation. However, this RNA is complexed as an RNA-DNA hybrid and thus is not available to bind to Rho. Nevertheless, if the 5' (RNA) end of the RNA-DNA hybrid contiguous to the advancing Rho hexamer melts transiently as a consequence of thermal fluctuations (amplified, perhaps, by a destabilization induced by the proximity of Rho itself), a portion of a correctly

positioned Rho binding site could interact with a transiently exposed segment of single-stranded RNA to initiate separation of the hybrid. Though the molecular details of this process remain to be elucidated, progress of the Rho hexamer into the RNA-DNA hybrid could, in principle, continue by such an "unzipping" mechanism until the RNA has been separated from the template DNA.

Relationship of Intrinsic to Rho-Dependent Termination. The above model also suggests that Rho-dependent and intrinsic transcript termination may represent thermodynamically comparable processes. For intrinsic termination, competitive invasion of the RNA-DNA hybrid of the elongation complex by the RNA termination hairpin may provide a major component of the free-energy change that destabilizes the elongation complex so that RNA release can occur (4). The Rho-dependent termination model presented here invokes the same thermodynamic idea, except that the complementary strand of the RNA hairpin involved in intrinsic termination is replaced by the RNA binding site of a Rho subunit, which also "invades" the RNA-DNA hybrid and competitively sequesters the RNA strand as a stable Rho-RNA complex. To make the hybrid separation reaction of Rho described above kinetically effective, an appropriate RNA binding site must be positioned correctly with respect to the RNA-DNA hybrid within the transcription complex. Protein-protein interactions between Rho and RNA polymerase could facilitate this alignment and increase the efficiency of the helicase action.

Conclusions

The physical model for Rho function presented here is based on several crucial features, each of which is strongly supported by structural and enzymatic data. These are (i) the functional unit at which the catalytic mechanism operates is the Rho dimer, (ii) the dimer is asymmetric and the orientation of the asymmetry can be reversed on the time scale of the catalytic cycle, (iii) adjacent RNA chain segments bind to the subunits of the dimer with opposite chain polarities, resulting in functionally different RNA binding sites, and (iv) the ATPase and the RNA binding and release cycles are coupled through the modulation of RNA binding affinity by conformational changes within the dimer. Less molecularly defined models for Rho function have been put forward previously and are briefly summarized here.

The observation that Rho and RNA polymerase mutants can complement one another to restore Rho-dependent termination has suggested that at least part of the functional basis of Rho action might involve direct interactions between Rho and the polymerase of the transcription complex (43). Clearly, such interactions could increase the efficiency of Rho helicase function, as suggested above. An extreme model suggests that RNA binding and ATPase activation simply "activate" Rho so that it can interact directly with the polymerase and trigger RNA release. This notion has motivated many attempts to induce Rho-dependent termination in "trans," usually by using poly(rC) to activate the ATPase of high concentrations of Rho molecules that are not directly bound to the RNA to be released. No induction of RNA release has been observed in such experiments.

Models in which the interaction between Rho and polymerase involves direct participation of the nascent RNA chain include "looping," "tracking," and "tethered tracking" proposals (for general descriptions of these models, see refs. 8 and 44). The model we describe here can be classified as a molecularly defined "pure" tracking model. Looping and tethered tracking models require that Rho exercise its RNA release function while remaining bound to its initial loading site on the RNA. In looping models, binding to the nascent RNA chain merely serves to increase the effective

local Rho concentration. Such models seem unlikely (8, 44). Tethered tracking models involve some form of tracking combined with continued binding of Rho to the initial loading site. Experimental support for such models includes the observation that this RNA sequence seems to be most protected in footprinting experiments on ATP-activated Rho-RNA complexes (45). In tracking models such as that described here, Rho is expected to scan and release each RNA chain repeatedly in the course of an experiment. Clearly, the initial loading site on the nascent RNA, which represents the equilibrium position of highest affinity for Rho, should also be the most highly occupied. Experiments designed to distinguish tracking and tethered tracking models will have to measure the rapid repartitioning of Rho between specific RNA sites as it translocates.

The hexameric Rho motif, including, where tested, suggestions of three strong and three weak ATP and perhaps nucleic acid binding sites, is found in a number of other nucleic acid helicases. Well-documented examples include the *E. coli* DnaB protein (46) and the large tumor antigen of simian virus 40 (47). It will be interesting to see whether other recently discovered helicases involved in cellular processes also exist as hexamers. However, other helicases seem to function as free asymmetric dimers (e.g., see ref. 48), so the hexameric motif may not be universal.

Note Added in Proof. It has been brought to our attention that a hexameric motif functionally resembling our Rho model has also been proposed for the F1 component of the ATP synthase complex (see ref. 50).

We are pleased to acknowledge helpful comments on this work from Terry Platt, John Richardson, Eric Steinmetz, and Mark Young. An earlier version of this model was submitted (by J.G.) to the Graduate School of the University of Oregon in partial fulfillment of the requirements for the Ph.D. degree in Chemistry (49). These studies were supported in part by U.S. Public Health Service Research Grants GM-15792 and GM-29158 (to P.H.v.H.) and by a grant to the Institute of Molecular Biology from the Lucille P. Markey Charitable Trust. P.H.v.H. is an American Cancer Society Research Professor of Chemistry.

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