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An RNA-Dependent Nucleoside Triphosphate Phosphohydrolase (ATPase) Associated with Rho Termination Factor

(*Escherichia coli*/RNA polymerase/transcription)

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ABSTRACT **Highly purified rho termination factor from *Escherichia coli* catalyzes an RNA-dependent hydrolysis of ribonucleoside triphosphates to nucleoside diphosphates and inorganic phosphate. In the presence of poly(C), a specific activity of 100 μmole of ATP hydrolyzed per min/mg has been measured. The phosphohydrolase activity appears to be associated with the protein responsible for termination of RNA synthesis, but a functional relationship between the two activities is not yet evident. Hydrolysis of nucleoside triphosphates occurs in the absence of termination and without any extensive degradation of RNA.**

The activity of the DNA-dependent RNA polymerase isolated from *Escherichia coli* can be modulated by several protein factors (1). One of these, called rho, causes termination of RNA synthesis at specific sites on both bacterial and bacteriophage DNA templates and release of the terminated RNA molecules (2–6). Although several possible models have been suggested to explain rho activity, very little is known about its mechanism of action. Rho protein binds to RNA, DNA, and RNA polymerase (3, 7–10), but it is not clear which, if any, of these binding reactions is functionally significant. It is known, however, that rho does not act as a ribonuclease on completed RNA molecules (2).

In order to understand more about the function and activity of rho in the reaction catalyzed by RNA polymerase, we have investigated the possibility that rho affects other aspects of RNA metabolism in addition to causing termination and release of RNA chains. In this paper we show that rho factor preparations catalyze an RNA-dependent hydrolysis of terminal phosphates from nucleoside triphosphates. Some basic characteristics of this reaction are presented along with preliminary evidence that it is catalyzed by the same protein responsible for termination. In addition, we present evidence that rho does not cause extensive exonucleolytic degradation of nascent RNA molecules. We thus rule out a model for rho action that involves continued RNA synthesis after the termination event coupled with immediate degradation of this RNA.

MATERIALS

The DNA-dependent RNA polymerase (nucleosidetriphosphate:DNA nucleotidyltransferase, EC 2.7.7.6) and rho were purified from *E. coli* B and *E. coli* MRE 600 as described previously (3). DNA from bacteriophages T4 and T7 were isolated by the method of Thomas and Abelson (11). [γ-^{32}P]-ATP was prepared by the method of Glynn and Chappell (12). T4 RNA was synthesized *in vitro* as described previously (3). Poly(C) was purchased from Miles. Marker yeast RNA for chromatography on Whatman DE-52 cellulose was digested with pancreatic RNase (Worthington) by the method of Takanami (13). ^{32}Pi, [^{14}C]ATP, [^{14}C]GTP, and [^{3}H]UTP were purchased from New England Nuclear Corp. [^{14}C]UTP and [^{14}C]CTP from Schwarz-Mann and polyethyleneimine-cellulose thin-layer sheets from Brinkman Instruments.

RESULTS

Requirements for Release of Pi from ATP. Preparations of rho factor catalyze the conversion of label from [γ-^{32}P]ATP to a form that is not adsorbed by active charcoal. This reaction requires RNA and is stimulated by $MgCl_2$ (Table 1). The RNA requirement is fulfilled well by poly(C). *In vitro*-synthesized T4 RNA also activates the ^{32}P release activity but is far less effective than poly(C) on an equal weight basis; it becomes more effective when higher amounts are used. T7 DNA,

TABLE 1. *Requirements for ATP hydrolysis*

Additional reactants	^{32}Pi released (nmole)
None	0.1
Poly(C)	18.0
Poly(C), RNase	<0.1
Poly(C), -$MgCl_2$	4.1
T4 RNA	0.7
Native T7 DNA	<0.1
Denatured T7 DNA	<0.1
RNA polymerase	0.1
Poly(C)-rho	<0.1

The basic reaction mixture contained 0.05 ml 0.04 M Tris·HCl (pH 8.0), 0.05 M KCl, 10^{-4}M EDTA, 10^{-4}M dithiothreitol, 12 mM $MgCl_2$, 0.3 mg/ml of bovine-serum albumin, 0.4 mM [γ-^{32}P]-ATP (1500 cpm/nmole), and 0.01 μg of rho. Additions where indicated were: 0.8 μg of RNA; 0.5 μg of RNA polymerase; 3.0 μg of T7 DNA; and 0.1 μg of pancreatic RNase. Denatured T7 DNA was prepared by heating native T7 DNA for 10 min at 100° in a solution containing 0.03 M KCl, 0.01 M Tris·HCl (pH 8.0), and 1 mM EDTA. The mixture containing RNase and poly(C) was incubated at 37° for 10 min with poly(C) prior to addition of [γ-^{32}P]ATP (when the RNase was added with the [γ-^{32}P]ATP, 2.2 nmole of ^{32}Pi was released). Following incubation for 30 min at 37°, ^{32}P not adsorbable by charcoal was determined by the method of Krakow (22) and counted by Cerenkov radiation (23). The moles of ^{32}Pi released is the amount above the 0.04 nmole of ^{32}Pi initially present in the [γ-^{32}P]ATP.

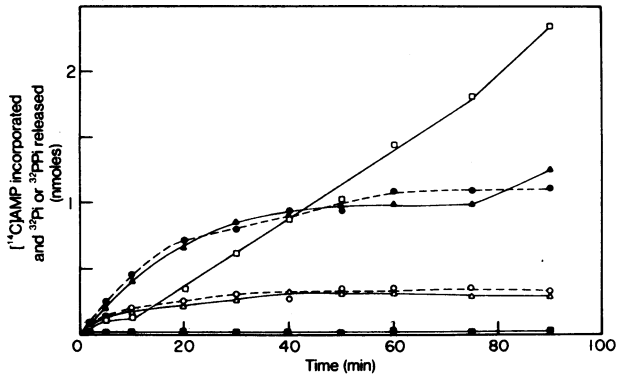

FIG. 1. Effect of rho on release of ^{32}Pi and ^{32}PPi from [γ-^{32}P]-ATP during *in vitro* transcription of T4 DNA. Each reaction mixture contained 0.04 M Tris·HCl (pH 8.0); 0.05 M KCl; 12 mM $MgCl_2$; 10^{-4} M EDTA; 10^{-4} M dithiothreitol; 0.3 mg/ml of bovine-serum albumin; 0.4 mM each of CTP, GTP, UTP, and [γ-^{32}P]- and [8-^{14}C]ATP (1500 cpm/nmole and 3,000 cpm/nmole, respectively); 0.3 μg of RNA polymerase; 1.8 μg of T4 DNA; and 0.10 μg of rho factor, where appropriate, in a final volume of 0.05 ml and was incubated at 37°. For measurement of the incorporation of [^{14}C]AMP into acid-insoluble material, the reaction was stopped by adding 0.5 ml each of 0.1 M $Na_4P_2O_7$ and 10% trichloroacetic acid, and the precipitated RNA was collected on Whatman GF/C filters and counted in a toluene-base scintillation fluid. For measurement of ^{32}Pi and ^{32}PPi release, material not adsorbed on charcoal was collected by the method of Krakow (21) and neutralized by addition of 0.25 ml of 1.0 M Tris. After application to a 30 × 5 mm Dowex AG1-X8 Cl column, the ^{32}Pi was eluted with 8 ml of a solution containing 0.01 N HCl and 0.05 M NaCl, and the ^{32}PPi was eluted with 15 ml of a solution containing 0.01 N HCl and 0.5 M NaCl. Radioactivity was determined as in Table 1. O——O, [^{14}C]AMP; □——□, ^{32}Pi; Δ——Δ, ^{32}PPi; *filled symbols*, without rho; *empty symbols*, with rho.

either denatured or native, and RNA polymerase alone do not replace RNA. Poly(C) alone has no activity, and its ability to stimulate the rho catalyzed activity is abolished by pretreatment with ribonuclease. Evidence that the released label is orthophosphate comes from its chromatographic properties on Dowex AG 1-X8 (chloride) (see Fig. 1 and 3) and its ability to form a complex with molybdate in acid solution that partitions into the organic phase upon extraction with an isobutanol-benzene mixture (14).

The requirement for RNA is also fulfilled by the RNA synthesized *de novo* in reaction mixtures containing rho, RNA polymerase, DNA, and the four ribonucleoside triphosphates (Fig. 1). To follow the ATP hydrolysis reaction in complete reaction mixtures, the ^{32}P released in each assay was chromatographed on a Dowex-1 (chloride) column to separate the ^{32}Pi from the ^{32}PPi also released as a product of the RNA synthesis reaction. In this experiment, the kinetics of ^{32}Pi release catalyzed by rho is compared with both the incorporation of [^{14}C]AMP into RNA and the release of ^{32}PPi in the presence and absence of rho factor. The ^{32}Pi release reaction starts after an initial lag of 8–10 min, which may reflect the time required to synthesize sufficient RNA to activate the hydrolase, and continues at a nearly constant rate for at least 80 min, or long after RNA synthesis has ceased. This experiment also shows the effect rho has on reducing the amount of RNA synthesized as measured by both

TABLE 2. *Hydrolysis of the four ribonucleoside triphosphates*

^{14}C-Labeled substrate	nmoles of nucleoside diphosphate produced
ATP	15.7
CTP	16.9
CTP	17.4
UTP	16.4

Reaction conditions were identical to those in Table 1 except that each mixture contained 0.5 μg of poly(C), one ^{14}C-labeled nucleoside triphosphate (10 μCi/μmole), and 0.01 μg of rho. After incubation for 15 min, the reaction was stopped by addition of 5 μl of 0.4 M EDTA and 5 μl of ice-cold 5 N perchloric acid. After removal of the precipitate by centrifugation at 7000 rpm for 7 min in the Sorvall SS-34 rotor, the supernatent fraction was neutralized with KOH and centrifuged again to remove the potassium perchlorate. A 25-μl sample of each was spotted onto polyethyleneimine sheets with carrier nucleoside diphosphates and triphosphates and chromatographed with LiCl solutions as described by Randerath (24). The separated spots, located with the aid of an ultra-violet lamp, were cut out and counted in a toluene-base scintillation fluid. The values given are corrected for the amounts of nucleoside diphosphate found after incubation of an identical mixture without rho factor. These values were: ADP, 1.3 μmole; CDP, 0.9 μmole; GDP, 0.6 μmole; and UDP, 0.9 μmole.

In an identical reaction containing [γ-^{32}P]ATP as the labeled substrate and run simultaneously, 14.5 nmole of ^{32}Pi was released.

nucleotide incorporation and ^{32}PPi release. It should be noted that the good agreement found with these two measurements indicates that the nucleotides added to nascent RNA in the presence of rho remain stably incorporated.

Formation of ADP. If Pi is one product of the ATP hydrolysis reaction catalyzed by rho, the other product should be ADP. The appearance of ADP is shown by ion-exchange chromatography of the acid-soluble products from a complete reaction mixture containing RNA polymerase, T4 DNA, rho factor, and the four nucleoside triphosphates with ATP labeled in the adenine ring with ^{14}C (Fig. 2). After 30 min, 45% of the remaining soluble adenine nucleotides is ADP (8% of the label was incorporated into RNA). The small amounts of label found in the peaks of adenosine and AMP are identical to the amounts found in a control reaction mixture incubated 30 min without rho. Initially less than 4% of the label was ADP, and this did not increase in the reaction without rho. This experiment shows, therefore, that ADP and not AMP or adenosine is the major product of the rho catalyzed reaction. A further proof that the major product of reaction is ADP is that alkaline phosphatase converts more than 97% of the labeled acid-soluble products to adenosine. Chromatography of the phosphatase treated nucleotides under conditions identical to those used in Fig. 2 showed that less than 1% of label in the ADP peak of Fig. 2 is from dinucleotides that elute at nearly the same place as ADP.

Evidence that ADP is a product of the simpler reaction mixtures that contain ATP, rho, and poly(C) has been obtained using thin-layer chromatography to separate the nucleotides (Table 2). This assay shows that the conversion of ATP to ADP is stoichiometric with the release of ^{32}Pi from [γ-^{32}P]ATP. When other nucleoside triphosphates are used instead of ATP, they are hydrolyzed by rho factor in the pres-

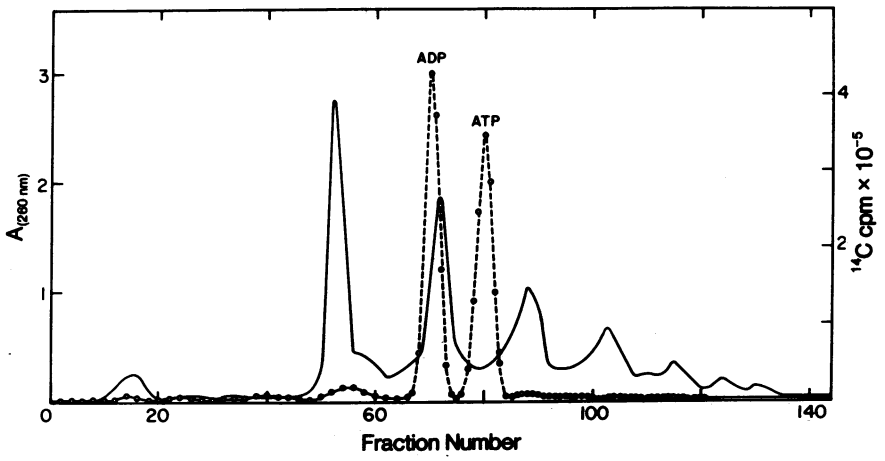

FIG. 2. Chromatography patterns of ^{14}C-labeled adenine nucleotides. Reaction conditions were identical to those in Fig. 1 except that 3.0 μg of RNA polymerase and 0.6 μg of rho were used; each NTP concentration was 0.2 mM; and the ATP was labeled only with ^{14}C (50 μCi/μmole). After incubation for 30 min, the reaction was stopped by addition of 10 ml of a solution containing 7 M urea and 0.05 M Tris·HCl buffer (pH 7.5) and 5 mg of marker RNA. The sample applied to a 50 × 1-cm DEAE-cellulose column was eluted with a 400-ml linear gradient from zero to 0.4 M NaCl in 7 M urea and 0.05 M Tris·HCl (pH 7.5). The flow rate was 70 ml/hr, and 2.0-ml fractions were collected. After chromatography, the A_{260} and radioactivity were determined (13). O——O; ^{14}C-labeled adenine nucleotides; —, A_{260}. (The numbers on the right ordinate have been multiplied by 10^{-5}.)

ence of poly(C) to the corresponding nucleoside diphosphates (Table 2). Under these conditions the rates of hydrolysis are nearly the same for each substrate. However, preliminary experiments indicate that with mixtures of all four substrates, ATP is strongly preferred. We suspect, therefore, that the hydrolase has a much stronger affinity for ATP; thus we call it an ATPase.

Evidence That Rho Protein Is Responsible for ATPase and Termination Activities. RNA-dependent ATPase activity has been found in three separate rho preparations: two from *E. coli* B and one from *E. coli* MRE 600. Although the preparations used for these studies were greater then 95% pure, as demonstrated by polyacrylamide gel electrophoresis, the possibility exists that the ATPase activity is catalyzed by a protein different from that responsible for rho termination activity. The following evidence suggests that one protein is responsible for both activities.

First, the two activities appear to have the same heat sensitivities. No distinction can be made in their rates of inactivation at 47° (Fig. 3; although the line drawn is that for a single first-order rate of inactivation, the activity measurements are too uncertain to exclude the possibility of more complicated kinetics).

Second, the two activities cosediment. Fig. 4 shows the profiles after zone sedimentation in 0.2 M KCl. Since the rho polypeptide is known to undergo a salt and concentration sensitive association (15), the trailing of activities found on this gradient is probably a result of dissociation that occurs during the sedimentation. The fact that both activities trail to the same extent is further evidence that they are functions of the same protein. When centrifuged in 0.05 M KCl, the two activities coincided in a single sharp peak with a sedimentation coefficient of 10 S.

DISCUSSION

ATPases are ubiquitous enzymes. The rho associated ATPase, however, is distinguished by its absolute requirement for RNA. Several DNA-dependent ATPases have been demonstrated and some of these may function with RNA as well (16–18), but since the rho ATPase is not active with DNA it is not one of the DNA-dependent enzymes. Recently it was shown that a complex of 5S RNA and proteins B-L5 and B-L22 from the 50S ribosomes of *Bacillus stearothermophilus* catalyze the hydrolysis of phosphates from ATP and GTP (19). The proteins in this complex, which are probably the ones responsible for the GTP hydrolysis steps of protein

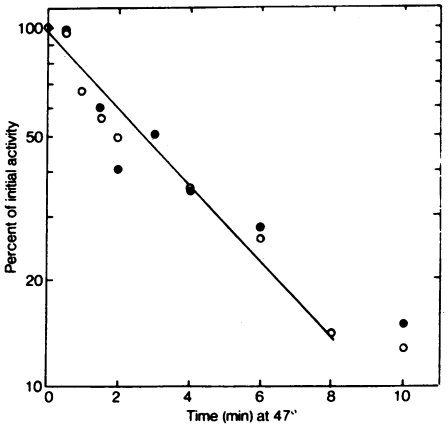

FIG. 3. Heat inactivation of ATPase and RNA termination functions of rho. Rho protein (100 μg/ml) was heated at 47° in a solution containing 10 mM Tris·HCl (pH 8.0), 10 mM MgCl, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1 mg/ml of bovine-serum albumin. Samples were removed at the times indicated and assayed for ATPase and termination activities. ATPase was assayed as described in Table 1 using 1.5 μg of poly(C). Termination activity was assayed by the capacity of the rho protein to inhibit transcription of T7 DNA under conditions identical to those in Fig. 1 except that the 0.1 ml final volume contained 4.0 μg of T7 DNA, 0.5 μg of RNA polymerase, 0.02 μg rho protein, and [^{3}H]UTP (10 μCi/μmole) as the labeled substrate. At t = 0, this much rho inhibited transcription by 45% (= 100% rho activity). ●, termination activity; O, ATPase activity.

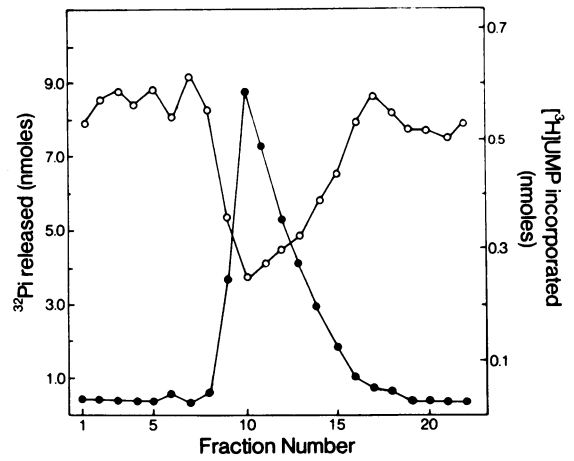

FIG. 4. Cosedimentation of rho factor and ATPase activities. Rho (20 μg) in 0.1 ml of a solution containing 0.2 M KCL, 100 μg/ml of bovine-serum albumin, and 0.05 M Tris·HCl (pH 7.4) was layered onto a 5-ml 10–30% glycerol gradient in the same solution. The sample was centrifuged in a SW50.1 Beckman rotor at 47,000 rpm for 16.5 hr and 0.20-ml fractions were collected by pumping from the bottom of the polyallomer tube. Reaction conditions for assaying inhibition of RNA synthesis were identical to those in Fig. 1 except that 0.1-ml assays contained 3.3 μg of T7 DNA, 0.1 μg of RNA polymerase, 10 μl of each gradient fraction, and [^{3}H]UTP (10 μCi/μmole). ATPase was assayed as described in Table 1 using 0.8 μg of poly(C) and 5 μl of each gradient fraction per assay. After incubation for 30 min at 37°, 1 ml of a solution containing 0.01 M Tris·HCl buffer (pH 8.0) and 0.01 M EDTA was added and the Pi was separated by chromatography on a Dowex AG1-X8Cl column as described in Fig. 1. O——O, [^{3}H]UMP incorporated; ●——●, ^{32}Pi released.

synthesis, could comprise another kind of RNA-dependent ATPase.

An important question posed by the finding of the ATPase activity in rho factor is whether it has some significance in the termination reaction. It is clear that ATP hydrolysis is readily uncoupled from termination; it is activated by RNA alone, and even under the condition when rho causes termination, the number of ATP molecules hydrolyzed exceeds the number of chains terminated by more than a thousand to one. On the other hand, it is not known whether the rho termination reaction depends on ATP hydrolysis.

It was shown previously that rho binds to RNA in the absence of nucleoside triphosphates (3). Although this fact is difficult to reconcile with the presumed mechanism for rho termination, it is a reasonable interaction for an RNA-dependent ATPase. The preliminary evidence concerning the nature of the RNA requirement suggests that sequence or structure is important; per unit weight, poly(C) is 20 times more effective an ATPase activator than T4 RNA.

Several of the DNA-dependent ATPases are also ATP-dependent DNases. These include some of the restriction endonucleases (16) and the DNase controlled by the *recB* and *recC* genes of *E. coli*. (17). In contrast, the existing evidence suggests that rho is not an ATP-dependent RNase. Roberts has shown that rho does not cleave or degrade large, isolated λ transcripts even in complete RNA polymerase reaction mixtures (2). From results presented in this paper it can be inferred that rho does not catalyze extensive degradation of nascent RNA molecules either. In Fig. 1, it is shown that at all times the amounts of ^{32}PPi released during RNA synthesis in the presence of rho is identical to the amounts of nucleotide incorporated into the RNA. If some RNA had been synthesized and subsequently degraded this would have been evident as a higher amount of ^{32}PPi released. The results in Fig. 2 also give no evidence for conversion of the adenine label of ATP to RNA cleavage products such as AMP or acid-soluble oligonucleotides. However, none of the experiment presented so far can eliminate the possibility that rho removes a few nucleotides from the 3′ end of each RNA molecule terminated by its action.

Although the evidence presented in this paper suggests that one protein is responsible for both termination and ATP hydrolysis, further evidence of physical and functional coincidence of these activities is needed to strengthen this conclusion. The specific activity for ATP hydrolysis of 100 μmol per min/mg (see Table 2) is similar to that of previously described ATPases: F_1 factor from beef-heart mitochondria has about the same activity (20) and the myosin ATPase has less than one fourth that activity (21). The fact that the level of activity is high is consistent with our belief that the rho protein—and not a minor contaminant—is responsible for the ATP hydrolysis.

Another question raised by the finding of this new activity is whether it reflects some other function of the rho protein. The amount of rho normally isolated from *E. coli* is about 0.4 mg/100 g of cells which is about 20 molecules per cell. This is a small number for a protein that presumably has an important role in transcription. The new activity may be useful in determining whether this low yield actually represents the amount of rho present in *E. coli* or whether a significant fraction is lost during purification. In addition, mutants of rho factor would be very useful in settling questions of its importance; the new activity could be used to screen for these mutants. Thus, although its function remains to be determined, the RNA-dependent ATPase activity should at least serve as a good alternative marker for this intriguing protein.

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