Conformational alterations of transcription termination protein rho induced by ATP and by RNA

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ABSTRACT

Transcription termination protein rho from *Escherichia coli* possesses an RNA-dependent ATP hydrolysis activity necessary for expression of its termination function. We have used the rate of trypsin-mediated inactivation of ATPase activity as a conformational probe to test for ligand binding-induced conformational changes in the rho polypeptide. When present in molar excess over rho polypeptide, trypsin inactivates rho ATPase by a first order process that correlates well with the loss of intact rho polypeptide. When rho protein binds poly(C) or poly(dC), its susceptible bonds become more accessible to trypsin action. On the other hand, when rho binds either ATP or ADP those bonds become less accessible. These results suggest that rho protein assumes an altered conformation when an RNA cofactor is bound and that it assumes a distinctly different conformation when a nucleotide substrate or product is bound. A special change in the accessibility of trypsin-susceptible bonds is also detected when rho in its complex with poly(C) is catalyzing the hydrolysis of ATP.

INTRODUCTION

The biosynthesis of RNA catalyzed by *Escherichia coli* RNA polymerase is regulated by the action of protein factors. One of these factors, rho, causes termination of transcription at specific sites on DNA templates (1, 2). Rho is also an RNA-dependent nucleoside triphosphate phosphohydrolase (NTPase, called an ATPase when ATP is the only nucleoside triphosphate present) (3) and its function in termination is dependent on its phosphohydrolase activity (4, 5).

Elongation of RNA chains during transcription occurs by a processive mechanism (6). Nascent RNA molecules are held very tightly by RNA polymerase molecules to most DNA sequences (7-9). However, spontaneous release of RNA occurs at a limited number of DNA sites, notably at rho-independent transcription termination sites (10, 11). At rho-dependent termination sites, on the other hand, RNA release does not occur spontaneously. Instead, action of rho is needed to mediate release of RNA from these sites (1), and this

action is driven by energy derived from the hydrolysis of nucleoside triphosphates (12, 13). The primary involvement of rho in transcription termination could be to act as an energy-dependent RNA release factor. This view is supported by the evidence that rho stimulates the release of RNA from isolated ternary transcription complexes by a reaction that is dependent on the presence of nucleotide substrates for rho-NTPase (12-14).

Like many other enzymes that catalyze the hydrolysis of NTPs, rho protein couples a chemical reaction - the hydrolysis of a phosphoanhydride - with a mechanical action - the release of an RNA molecule. Studies of the effects of ATP hydrolysis on the accessibility of the ends of poly(C) and poly(U,C) molecules complexed with rho indicates that the chemical reaction is coupled to a conformational change in the RNA bound to rho (15). Since proteins do not have rigid structures, the mechanical action of rho could be a result of conformational transitions in the protein itself associated with changes in the molecules bound in the active site when an NTP is hydrolyzed to an NDP and Pi; rho protein could have one conformation when ATP is bound and another when ADP is bound or one conformation when a nucleotide is bound and another when the nucleotide has dissociated.

An example of a conformational transition dependent on nucleotide binding has been demonstrated for the protein synthesis elongation factor Tu (EF-Tu), a protein that has a functional GTPase activity. Douglass and Blumenthal (16) showed that when EF-Tu contains a bound GTP, its peptide backbone is cleaved more rapidly by action of trypsin than when it contains a bound GDP. The rate of cleavage of the region of a protein that is most accessible to a protease such as trypsin is a sensitive probe for a conformational alteration because such an alteration is likely to involve changes in the local folding of the protein in an exposed, hinge-like region. Based on their results with EF-Tu, Douglass and Blumenthal proposed that a hinge-like region might be a common structural feature of proteins that convert chemical energy to mechanical energy by catalyzing the hydrolysis of NTPs. Hence, analysis of the effects of reaction components on the rates of cleavage of other NTPases could provide useful insights into the mechanism of action of such enzymes.

In this paper we use the kinetics of cleavage of rho protein by trypsin as a probe for conformational transitions associated with its ATPase activity. We establish first that action of trypsin on rho protein induces loss of ATPase activity with the same rate as the appearance of the first cut in the rho polypeptide. We thus use ATPase activity measurements to analyze the

effects of various ligands on the rate of trypsin cleavage of rho. As a model for the RNA cofactor we use poly(C), which is a potent ATPase activator and can bind to rho protein very tightly (15). The results presented here reveal three distinct changes in the susceptibility of rho protein to trypsin action: one change is caused by binding of the RNA cofactor, another by binding of a nucleotide substrate or products of the ATP hydrolysis reaction, and the third is associated with the dynamic process of ATP hydrolysis.

MATERIALS AND METHODS

Chemicals and Biochemicals. ATP, trypsin (bovine pancreas) and trypsin inhibitor (soybean) were purchased from Boehringer-Mannheim Biochemicals. ADP was obtained from Sigma Chemical Corp. [γ - ^{32}P]ATP was prepared by the method of Schendel and Wells (17) using [^{32}P]H $_3$ PO $_4$ purchased from ICN Pharmaceuticals Inc. Poly(A), poly(C), poly(dC), adenosine- β - γ -methylene triphosphate were purchased from Miles Biochemicals. Acetylated bovine serum albumin was from Bethesda Research Laboratory. All other chemicals were reagent grade. Rho protein was purified from *E. coli* MRE 600 by the method of Finger and Richardson (18).

Trypsin cleavage. To follow the kinetics of digestion of rho with trypsin, a reaction mixture containing 9.3 μg rho and 12 μg trypsin in 100 μl of a solution containing 0.04 M Tris-HCl (pH 8) 0.05 M KCl; 1 mM MgCl $_2$; 0.1 mM EDTA; 0.1 mM dithiothreitol was incubated at 25°C. Samples of 10 μl removed at various times were added to 10 μl of a 0.1% solution of trypsin inhibitor on ice. Control experiments showed that when trypsin was added to a solution of rho and trypsin inhibitor at the concentrations used in these experiments, there was no cleavage of the rho after 30 min at 25°C.

ATPase assays. To determine the amount of active rho-ATPase remaining in the digested samples, 2 μl portions were assayed in standard rho-ATPase reaction mixtures that contained 0.04 M Tris-HCl (pH 8), 0.05 M KCl, 1 mM MgCl $_2$, 0.1 mM dithiothreitol, 0.1 mg acetylated bovine serum albumin/ml, 5 μg poly(C)/ml and 1 mM [γ - ^{32}P]ATP in a total volume of 0.1 ml. After incubation for 10 min at 37°C the amount of ^{32}Pi release was determined as described previously (19). Control experiments showed that the presence of either trypsin inhibitor or trypsin plus trypsin inhibitor did not inhibit rho ATPase.

Gel electrophoresis. To prepare digested rho for analysis by gel electrophoresis, an 18 μl portion of a sample from the digestion reaction

Fig. 1. Time course of cleavage and inactivation of rho by action of trypsin. The reaction mixture contained 9.3 μ g rho protein and 12 μ g of trypsin in 100 μ l and was incubated at 25°C. Samples removed at the indicated times were analyzed for ATPase activity and amount of 46 kDa protein (rho) as described in the Material and Methods section. (●) ATPase activity (○) 46 kDa rho protein.

quenched with trypsin inhibitor was mixed with 4.6 μ l of a 5-times concentrated stock solution of the final sample buffer described by Laemmli (20) and this mixture was heated 1 min at 90°C. Each sample was applied to an individual well of a 1.5 mm thick slab gel consisting of 1 cm of a 3% polyacrylamide stacking gel and 9 cm of a 10% polyacrylamide running gel in the buffer system described by Laemmli. After electrophoresis, the gels were stained with Coomassie brilliant blue, destained and scanned in a Helena Quick-Scan microdensitometer. The areas under peaks were integrated with a planimeter.

RESULTS

Cleavage of rho at its most susceptible bonds inactivates its ATPase activity. When rho protein was digested with trypsin (2.6 moles trypsin/mole of rho monomer), it lost ATPase activity by a first order process with a half-life of 9.2 min (Fig. 1). This rate was at least 7 times faster than the rate of rho-inactivation when rho protein was incubated under the same conditions in the absence of trypsin (Table 1). To determine the relationship between loss of activity and the cleavage of rho by trypsin, samples

Table 1
*Effects of nucleotides and nucleic acids on the rates
 of inactivation of rho ATPase by trypsin*

Nucleotides and nucleic acids added	Half-life for loss of rho activity (min)
none (free rho)	1.3 $\pm$ 0.2
ATP	3.4 $\pm$ 0.3
ADP	3.0 $\pm$ 0.3
App(CH ₂)p	3.5 $\pm$ 0.3
poly(C)	0.5 $\pm$ 0.1
poly(A)	1.6 $\pm$ 0.2
poly(dC)	0.5 $\pm$ 0.1
poly(C), App(CH ₂)p	1.75 $\pm$ 0.2
poly(C), ADP	1.6 $\pm$ 0.2
poly(dC), ATP	1.6 $\pm$ 0.2
poly(C), ATP	2.5 $\pm$ 0.2
none, no trypsin	65 $\pm$ 7

The data are from the analysis of the curves shown in Fig. 3 plus curves from similar experiments not shown. In all cases, the conditions for digestion and assay were as described in the legend for Fig. 3. Indicated polynucleotides and nucleotides were 50 μ g/ml and 1 mM, respectively.

were removed from the digestion mixture at various times and analyzed by gel electrophoresis. Rho protein, which has $M_r = 46,094$ (21), is well resolved from trypsin ($M_r = 23,800$) and trypsin inhibitor ($M_r = 21,000$) in the Laemmli gel system (Fig. 2). The results show that the intensity of the intact rho band decreased steadily with time of digestion. The amounts of intact rho polypeptide remaining were determined by scanning the stained band on the gel with a recording densitometer. A plot of the area of the peak as a function of digestion time (Fig. 1) shows that the rate of loss of the intact polypeptide was the same as the rate of loss of rho ATPase activity. The coincidence of these rates suggests that a single break in a rho

Fig. 2. Trypsin cleavage of rho protein. Samples removed at the indicated time from the digestion reaction described in Fig. 1 (lanes 3-9) plus control samples of rho alone (lane 1) and rho with trypsin inhibitor (lane 2) were subjected to polyacrylamide gel electrophoresis as described in the Material and Methods section. The indicated bands are: rho, rho protein; 32 k, 32 kDa fragment; tryp, trypsin; t.i., trypsin inhibitor.

polypeptide chain at a site accessible to trypsin action is sufficient for complete loss of the ATPase activity of that polypeptide.

The gel electrophoretic analysis shows too (Fig. 2) that partial digestion gave a major fragment with $M_r \approx 32,000$ (band labeled 32k). This fragment was sensitive to further digestion and was cleaved with a rate that was similar to or greater than the rate of cleavage of intact rho.

Use of trypsin-cleavage as a probe of protein conformation. In many cases, changes in the conformation of a protein will alter the rate with which a protease can act to cleave susceptible bonds. Since loss of rho ATPase activity correlates with the extent of cleavage of rho protein, a measurement of the rate of loss of activity should give, indirectly, the rate of cleavage. To test whether the binding of cofactors and/or nucleotide substrates alter the conformation of rho protein, we measured the effects of these substances on rates of trypsin-mediated inactivation of rho-ATPase. Results presented in Fig. 3 and also summarized in Table 1 show that when 1 mM ATP was present with rho, the rate of trypsin-mediated inactivation was 0.4 times as fast as when rho was alone. Since ATP did not affect the activity of trypsin with a simple substrate, benzoyl-L-arginine ethyl ester

Fig. 3. Time course of trypsin-mediated inactivation of rho alone (O) and of rho in complexes with ATP (■), poly(C) (●) plus ATP (▲). Trypsin cleavage was performed and ATPase activity assayed as described in the Material and Methods section except that the digestion mixture contained 25 μ g rho protein and 100 μ g trypsin in 0.5 ml. Poly(C) was 50 μ g/ml and ATP was 1 mM, when added. Under these conditions, the supply of ATP was sufficient to last 5 min. in mixtures with rho and poly(C).

(data not shown), we conclude that ATP affects the structure of rho protein rather than the catalytic activity of trypsin itself. When ADP was present instead of ATP, the rate of inactivation was nearly the same as it was with ATP, i.e. about 0.4 times the rate for rho alone. This result suggests that rho is in the same basic conformation when it is bound to the nucleotide product of the hydrolysis reaction as when it is bound to the substrate. The effects of ATP and ADP on trypsin action were also verified with the gel electrophoresis assay for intact rho polypeptides. In both cases, the rate of cleavage agreed perfectly with the rate of inactivation, and partial digestion again yielded a major fragment with $M_r \approx 32,000$ (data not shown).

Hypersensitivity of rho in the rho-poly(C) complex. Fig. 3 shows that the binding of poly(C) to rho had the opposite effect on its trypsin sensitivity as did the binding of ATP or ADP; rho was inactivated 2.6 times as fast by trypsin action with poly(C) present than with poly(C) absent. A significant enhancement of the rate of cleavage with poly(C) present was verified by gel electrophoresis. Again, since poly(C) did not change the activity of trypsin with a simple substrate, we conclude that the presence

of a poly(C) molecule tightly bound to rho increases the accessibility of the susceptible sites on rho protein. Partial digestion of rho with poly(C) present also yielded a 32 kDa fragment as a major product (data not shown). Thus, the hypersensitivity of rho in its complex with poly(C) is not a consequence of the exposure of a new cleavage site in the domain of that sub-fragment.

Since rho protein becomes part of a larger complex when it is bound to poly(C), the presence of the added macromolecule could serve as an "antenna" to increase the rate of formation of a productive contact between rho protein and trypsin. However, because poly(A), which can also bind to rho albeit less tightly than poly(C), did not alter the rate of trypsin-mediated inactivation (Table 1), we infer that the presence of a bound RNA by itself is not sufficient to accelerate the trypsin cleavage process. Thus, we favor the interpretation that formation of the tight complex with poly(C) causes a change in the conformation of rho protein that makes the trypsin sensitive site(s) more accessible. Table 1 also shows that a similar change occurs when rho forms its very stable complex with the cofactor analogue poly(dC).

Since rho catalyzes the hydrolysis of ATP to ADP in the presence of poly(C), it is not possible to prepare a stable complex of ATP bound to rho-poly(C). However, analogues of ATP have been prepared that can mimic the binding of ATP to proteins but are not hydrolyzed by ATPases. One analogue of this type is adenosine 5'- β , γ -methylene triphosphate (App(CH₂)p). The binding of App(CH₂)p to free rho protein appeared to cause the same conformational change as the binding of ATP; the rate of trypsin-mediated inactivation was the same with 1 mM App(CH₂)p as with 1 mM ATP (Table 1). When App(CH₂)p was added to the rho-poly(C) complex, the half-life for trypsin-mediated inactivation increased 3.5 fold (from 0.5 min for rho-poly(C) to 1.75 min for rho-poly(C) plus 1 mM App(CH₂)p). Table 1 also shows that ADP had a similar effect. Since these increases in resistance were similar to the proportionate increases in the half-life of rho protein with App(CH₂)p or ADP (half-lives = 3.5 min and 3.0 min) over rho protein alone (half-life = 1.3 min), we infer that the conformational change caused by the binding of these adenine nucleotides is independent of the conformational change caused by the binding of poly(C).

Even though it is not possible to prepare a stable complex of ATP bound to the rho-poly(C) complex, it is possible to measure the rate of trypsin-mediated inactivation of rho when ATP is added to the rho-poly(C) complex.

We consistently and reproducibly found that rho protein with poly(C) was more resistant to inactivation in the presence of ATP than it was in the presence of either App(CH₂)p or ADP (Table 1). However, when ATP was added to the complex of rho protein with poly(dC), the rate of inactivation was the same or nearly the same when ADP or App(CH₂)p were added to the complex of rho protein with poly(C). Since no ATP was hydrolyzed with the rho-poly(dC) complex, we conclude that the higher resistance in the rho-poly(C) complex compared to the rho poly(dC) complex caused by ATP is a consequence of a change in structure that is related to the hydrolysis reaction.

DISCUSSION

We have shown that action of trypsin on rho protein inactivated its ATPase activity with the same first-order kinetics as the cleavage of the rho polypeptide and that a major product of digestion was a fragment with $M_r \approx 32,000$. We also showed that binding of various substrates, products, substrate analogues, cofactors and cofactor analogues perturb the rate of the trypsin-catalyzed inactivation process. Since these perturbants do not appear to affect trypsin itself, we conclude that changes in the rate of inactivation are due to conformational alterations in rho protein or in the complex between rho protein and RNA that affect the accessibility of the proteolytic enzyme to the susceptible bonds in rho protein.

The binding of polynucleotide cofactors and nucleotides appear to cause two distinctly different conformational alterations. When rho binds to an RNA cofactor or a cofactor analogue (poly(dC)), it apparently assumes a more sensitive configuration, and when it binds nucleotides, it assumes a more resistant configuration. A change in conformation associated with the binding of poly(C) could be related to the activation of the rho ATPase by RNA. Although free rho can bind ATP tightly, a conformational change in the protein may be needed to align the functional groups that act to catalyze the hydrolysis of the γ -phosphoryl group of the ATP. However, the fact that poly(dC) induced the same change in trypsin sensitivity as poly(C) suggests that the conformational change alone is not a sufficient condition for ATPase activation. Although poly(dC) is not a cofactor by itself, it does act as a cofactor component in combination with short segments of RNA (22). Thus, its function as a partial cofactor may be to induce the conformational alteration in rho protein, while some other interaction with RNA provides the function to complete the activation process.

Conformational transitions associated with the binding and release of

nucleotides may also be what allows rho to terminate the transcription of an RNA. Rho protein apparently can couple ATP hydrolysis with an action on an RNA transcript that dissociates the transcript from its complex with RNA polymerase. In one model that has been proposed (15), the action of rho on an RNA molecule is mediated by periodic alterations in the affinity of RNA for one of two distinct RNA binding sites in a rho polypeptide. The results of the trypsin sensitivity studies indicate that rho protein is in one conformation when either ATP or ADP is bound and in another conformation when no nucleotide is bound. Thus, the catalytic cycle of ATP hydrolysis could cause a rho polypeptide to make orderly transitions between those two conformations. The binding of ATP to a subunit would force a conformational change that converts an RNA binding site on that subunit to its high affinity state. Hydrolysis of the ATP converts it to ADP, which does not bind as tightly to rho as does ATP (C. Andrews and J.P.R. unpublished results); hence the rapid dissociation of ADP would allow the rho subunit to isomerize back to its original conformation with the RNA binding site in its low affinity state.

In most cases, the effects of RNA and nucleotides on the trypsin sensitivity of rho were independent of each other. An important exception was found when the combination of polynucleotide and nucleotide allows the nucleotide to be hydrolyzed by rho action, as when rho is mixed with poly(C) and ATP. In this case, rho protein was significantly more resistant to trypsin action than in the cases when nucleotides were not being hydrolyzed. Thus, either a rho polypeptide assumes a more resistant conformation when it goes through the process of ATP hydrolysis or changes in the interaction between rho and RNA that are coupled to ATP hydrolysis make rho protein less accessible to trypsin.

The fact that the trypsin-mediated loss of rho ATPase activity follows the same first order kinetics as the initial cleavage of rho protein suggests that the inactivation event is a single hit process. In the simplest interpretation, a single break in the backbone of rho protein is apparently sufficient to abolish the activity contribution of that polypeptide. This is an interesting result because rho is known to act as a hexameric protein (23) that may require cooperation between the subunits. Thus, in order to prevent the loss of a cooperative effect by the presence of a damaged subunit in the rho hexamer, there may be a constant process of subunit exchange that will allow a cleaved, inactive subunit to be replaced by an intact subunit from another hexamer or from an intermediate in the assembly or dis-

assembly of a hexamer.

In a parallel and independent study, Bear *et al.* (24) found that limited trypsinolysis of rho protein in its complex with poly(C) and ATP produced fragments with $M_r \approx 31,000$ and 15,000. We failed to detect the 15 kDa fragment because our gel electrophoresis conditions did not resolve proteins with $M_r < 20,000$ and the trypsin inhibitor contained peptides in that size range. In addition, their results concerning the stabilization of rho by ATP or ADP and its destabilization by poly(C) or poly(dC) agree with ours. However, they did not observe the similarities in the rates of ATPase inactivation and of cleavage of rho polypeptide that we describe in Figs. 1 and 2. Instead they found that limited trypsinolysis of rho protein in a mixture with poly(C) and ATP caused ATPase activity to decrease more rapidly than the amount of intact rho polypeptide. One possible reason for this discrepancy is that their conditions for cleavage are less favorable for subunit exchange. Our measurement of the two rates were done in the absence of poly(C), and poly(C) is known to stabilize the hexameric form of rho protein (23). Hence, Bear *et al.* might have been detecting the deleterious effect of the presence of inactive subunits for the rho hexamer while under our conditions a rapid subunit exchange might have allowed an assembly of all remaining intact subunits in fully functional hexamers. Experiments are currently in progress to determine the effects of defective subunits on the activity of a hexamer and the effects of conditions on the rates of subunit exchange.

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