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Escherichia coli transcription termination factor ρ has a two-domain structure in its activated form

(RNA-dependent ATPase/protein domains/RNA–protein interactions/proteolysis/hinge region)

DAVID G. BEAR*†, CHRISTOPHER L. ANDREWS†, JEFFREY D. SINGER*†, WILLIAM D. MORGAN*‡, RAYMOND A. GRANT§, PETER H. VON HIPPEL*, AND TERRY PLATT§¶

*Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, OR 97403; §Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510; and †Department of Cell Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131

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ABSTRACT **Limited tryptic digestion of *Escherichia coli* transcription termination factor ρ [an RNA-dependent nucleoside triphosphatase (NTPase)] yields predominantly two fragments (f1 and f2) when the protein is bound to both poly(C) and ATP. The apparent molecular masses of the two fragments are 31 kDa for f1 and 15 kDa for f2, adding up to the molecular mass of the intact ρ polypeptide chain (46 kDa). Sequence analysis of the amino termini demonstrates that f1 is derived from the amino-terminal portion of ρ and that the trypsin cleavage that defines f2 occurs at lysine-283. These results suggest that, in the liganded (activated) form, the native ρ protein monomer is organized into two distinct structural domains that are separable by a single proteolytic cleavage. The f1 fragment, purified from NaDodSO$_4$/polyacrylamide gels and renatured, binds poly(C) but the f2 fragment does not; neither regains any ATPase activity. ATP- and polynucleotide-dependent changes in the rate of proteolysis and in the character of the fragments produced suggest that ρ undergoes a series of conformational transitions as a consequence of RNA binding, NTP binding and NTP hydrolysis. The rate of loss of ρ ATPase activity and of intact ρ monomers is slower in the presence of adenosine 5′-[γ-thio]triphosphate than in the presence of either ATP or ADP, indicating that the hydrolysis of ATP may result in different conformational effects than does the binding of this ligand. These findings are discussed within the context of recent models of ρ-dependent transcription termination.**

Transcription termination factor ρ catalyzes the site-specific release of selected transcripts from the genomes of *Escherichia coli* and coliphages (see refs. 1–3 for recent reviews). The ρ factor is a hexameric protein composed of six individual subunits of 46 kDa each (4, 5) and has an RNA-dependent nucleoside triphosphatase (NTPase) activity that is maximally stimulated *in vitro* by the binding of single-stranded cytidine-containing RNA (6, 7); interaction with the ternary transcription complex also stimulates the ρ NTPase activity (8–10). Physical contact between ρ and RNA polymerase during transcription termination is suggested by genetic evidence (8, 11, 12), but this contact may not be direct (41). However, the locations on the ρ protein of the recognition and binding sites for ATP, RNA, and various components of the transcription complex have not been established, and the interactions that occur within the native hexamer during transcription termination are not known.

Numerous nucleic acid binding proteins, as well as ATPases, have been shown to be separable into functional domains by limited proteolytic digestion (13–21). We therefore decided to try a similar approach for ρ factor to determine whether proteolytic dissection would reveal functionally discrete domains. In addition, if detected, changes in digestion of ρ under various conditions and in the presence of various ATPase substrates and effector ligands might yield insights into possible relationships between changes in domain organization of the ρ monomer and the mechanism of ρ-dependent transcription termination. Our results show that the active ρ hexamer has a well-defined domain structure for each ρ monomer when bound to RNA and ATP. Significant differences in proteolytic cleavage of ρ are seen in the absence of either or both of these ρ-binding cofactors/substrates. Thus, this "proteolytic probe" technique supports the notion that ρ undergoes a conformational change upon activation of the ATPase, consistent with models in which conformational changes are involved in the mechanism of ρ-dependent transcription termination.

MATERIALS AND METHODS

Buffers. TDMK buffer contained 40 mM Tris Cl (pH 7.5), 0.1 mM dithiothreitol, 5 mM $MgCl_2$, and 50 mM KCl. ρ storage buffer contained 50 mM Tris Cl (pH 7.7), 50% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 100 mM KCl.

Nucleotides and Proteins. TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin was obtained from Worthington; nucleotides and polynucleotides were from P-L Biochemicals. ρ protein was purified from *E. coli* strain MRE600 by the method of Wu *et al.* (22). Final purification on a heparin-agarose column (0.2–0.8 M KCl gradient elution) removes most of the minor contaminants that are carried through the initial purification procedure.

Proteolytic Digestion and Gel Electrophoresis. Approximately equal volumes of a 1 μg/μl solution of ρ (in storage buffer) and TDMK buffer were mixed together in a 1.5-ml microcentrifuge tube. For analytical studies, the solution was then distributed as 10-μl aliquots. One microliter of poly(C) (1 mg/ml in TDMK buffer), and/or 1 μl of 0.01 M ATP (in TDMK buffer and previously neutralized with NaOH) were added to the appropriate samples. One microliter of freshly made trypsin solution (0.1 mg/ml in H_2O) was added and the tubes were incubated at 37°C for 60 min. Control experiments (see *Results*) in which ATP was added periodically to the RNA-activated ρ showed no effect on the size of the fragments produced in the digestion reaction, indicating that depletion of ATP during the reaction was not a problem. The reaction was stopped by addition of NaDodSO$_4$/urea sample buffer (6 M urea/5% 2-mercapto-

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Abbreviation: ATP[S], adenosine 5′-[γ-thio]triphosphate.

‡Present address: Department of Biochemistry, University of California at Berkeley, Berkeley, CA 94720.
¶Present address: Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642.

ethanol/2% $NaDodSO_4$/10% glycerol/125 mM Tris Cl, pH 6.8) and heating for 5 min at 90°C. The mixture was electrophoresed, using the buffer system of Laemmli (23), in 1.5-mm thick $NaDodSO_4$/urea/polyacrylamide gels (5% acrylamide stacking gel, 15% acrylamide separating gel) at 50 volts for approximately 15 hr. Proteins were visualized by Coomassie blue or silver staining (24).

Renaturation, Filter Binding, and ATPase Assays. Uncleaved ρ polypeptide chains and the f1 and f2 fragments were extracted from polyacrylamide gels and renatured according to the procedure of Hager and Burgess (25). The final protein concentration for each sample was adjusted to 2.5 μM (1.2 mg/ml for intact ρ). For filter binding assays, the fragments were mixed with 500 ng of [^{3}H]poly(C) (0.4 μCi/ml; 1 Ci = 37 GBq) and 0.1 mM ATP, in a final volume of 100 μl in TDMK buffer. After incubation at 37°C for 20 min, each sample (12,000 cpm total) was filtered on a prewashed (with buffer) 24 mm HAWP Millipore filter, rinsed twice with 100 μl of TDMK buffer followed by 100 μl of 100% ethanol, dried, and assayed for radioactivity by liquid scintillation counting in 7 ml of New England Nuclear 963 cocktail. ATPase assays were carried out by either of two methods, as described previously (7, 26).

RESULTS

Partial Tryptic Digestion of ρ Yields Two Major Fragments Derived from Separate Regions of the ρ Polypeptide. ρ protein was digested with trypsin in the presence or absence of polynucleotides and ATP. Limited digestion of ρ protein alone yields a number of fragments (Fig. 1, lane 2). The presence of poly(C) during the digestion greatly enhances the proteolysis of the ρ protein (lane 3); the major final product of extensive digestion in the presence of polynucleotide is a single fragment of 14–15 kDa. The presence of ATP during the digestion greatly diminishes the extent of tryptic cleavage (lane 7). The addition of both ATP and poly(C) promotes the generation of two dominant fragments of 31 kDa (f1) and 17 kDa (f2) (lane 4) in addition to some minor fragments.

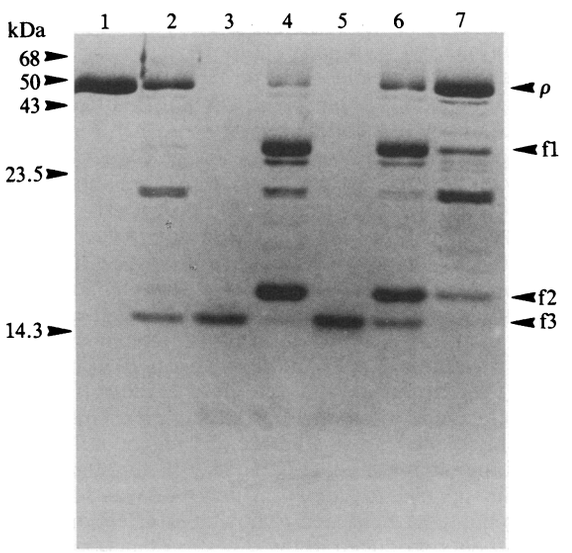

FIG. 1. Digestion of ρ with trypsin in the presence and absence of polynucleotides and ATP. Each reaction mixture (lanes 2–7) contained 1.3 μg of ρ protein and 0.1 μg of trypsin in 13 μl of TDMK buffer and was incubated for 1 hr at 37°C. After $NaDodSO_4$/PAGE, proteins were visualized by silver staining. Lanes: 1, ρ without trypsin; 2, ρ; 3, ρ + poly(C); 4, ρ + poly(rC) + 1 mM ATP; 5, ρ + poly(dC); 6, ρ + poly(dC) + 1 mM ATP; 7, ρ + 1 mM ATP. Positions of standards are indicated at left, and those of ρ and its fragments f1–3, at right.

The generation of fragments f1 and f2 is not dependent upon the hydrolysis of ATP during the tryptic digestion. ADP and adenosine 5'-[γ-thio]triphosphate [ATP[S], an ATP analog that inhibits ρ ATPase activity (26)] produce qualitatively the same digestion pattern as ATP (although the rates of digestion and loss of ATPase activity differ—see below), whereas AMP seems to have no effect on the digestion (data not shown). Although the polydeoxyribonucleotide poly(dC) does not stimulate ρ ATPase activity (27), it binds tightly to ρ protein (28) and can substitute for poly(C) in conjunction with ATP to stimulate the two-fragment proteolytic digestion pattern (Fig. 1, lane 6). Other polyribonucleotides, such as poly(A), give the same result at concentrations identical to that of poly(C) used in the above experiments. Control experiments, in which bovine serum albumin was treated with trypsin in the presence of various concentrations of ATP, indicate that under our buffer conditions the inhibitory effect of ATP on the rate of proteolytic digestion of ρ cannot be explained by direct inhibition of the trypsin by ATP (data not shown).

To determine the origins of f1 and f2, amino acid sequences of the amino-terminal regions were obtained by analysis of 300 μg of trypsin-treated ρ after separation of undigested ρ, f1, and f2 in $NaDodSO_4$/polyacrylamide gels by the method of Weiner *et al.* (29). A weak methionine signal for the amino terminus (not unusual, due to multiple oxidation products) was followed by aspartic acid (for asparagine), leucine, and threonine for the next three amino acids of both intact ρ and f1, in agreement with the previously determined sequence (5, 30). Similar analysis of the fragment f2 yielded the amino-terminal sequence Val-Leu-Thr-Gly, which aligns perfectly and uniquely with the predicted sequence following Lys-283 (5). These results are summarized in Fig. 2.

The apparent molecular masses of f1 and f2, as judged by $NaDodSO_4$/PAGE, are very close to those that would be generated by a single cleavage at Lys-283. The molecular mass of f1 predicted from the amino acid sequence is 31 kDa, while that predicted for f2 is 15 kDa (about 2 kDa smaller than the apparent molecular mass of this fragment on the gel). It is therefore likely that f1 comprises the first 283 amino acids, and f2 the remaining 136 amino acids of the ρ polypeptide chain, although we cannot rule out the possibility of limited additional degradation at the carboxyl terminus of either fragment.

The above amino-terminal analyses were carried out on ρ protein purified from MRE600, a wild-type strain of *E. coli* (31). The DNA sequence for ρ has only been determined for the *E. coli* K-12 ρ gene (5). We believe that the ρ proteins isolated from these strains are identical, or very nearly so, for the following reasons. The amino acid composition determined for purified MRE600 ρ protein and that predicted from the K-12 DNA sequence are essentially identical (4, 5). Furthermore, all other properties of the two proteins that have been compared are indistinguishable, including tryptic digestion patterns, electrophoretic mobility, isoelectric point, polynucleotide binding, kinetic parameters, and behavior in transcription systems *in vitro* (refs. 4, 5; unpublished re-

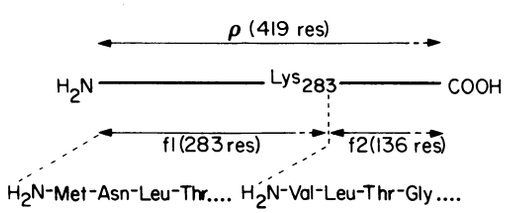

FIG. 2. Identification of the primary locus of cleavage of ρ monomer by trypsin in the presence of poly(C) and ATP (see text). res, Amino acid residues.

sults). It should be noted here that a previous designation of MRE600 as an *E. coli* B strain (4, 5) was incorrect (J. P. Richardson, personal communication).

Proteolytic Cleavage Eliminates the ATPase Activity of ρ. To determine whether proteolytic fragments f1 and f2 can function as ATPases, either alone or in association with one another, the fragments were extracted from the $NaDodSO_4$/polyacrylamide gels, and renaturation was attempted by the procedure of Hager and Burgess (25). As a control, undigested ρ was recovered from the gels and subjected to the renaturation protocol. We were able to recover 50–70% of the original specific ATPase activity from renatured intact ρ, in agreement with previous results of others (25). However, neither f1 nor f2 alone, nor f1 and f2 renatured together under the same conditions, displayed any poly(C)-dependent or poly(C)-independent ATPase activity (data not shown).

Proteolytic Fragment f1 Carries an RNA Binding Site. Even though neither renatured fragment f1 nor f2 (separately or together) show any RNA-dependent ATPase activity, we wished to determine whether either fragment can bind RNA. Fig. 3 shows that renatured f1, under conditions of moderate ionic strength, does indeed bind to poly(C) with an affinity comparable to that of intact renatured ρ. Fragment f2 shows no significant RNA binding. Although the latter negative result could simply reflect either the failure of gel-extracted fragment f2 to refold into a conformation similar to its structure in the native intact protein or the presence of a partial site on f2 too weak to be detected under these conditions, these results do suggest that most of the RNA binding to ρ protein is mediated by the f1 domain and that this fragment can renature to regenerate an active RNA-binding site.

To investigate which domain of the ρ protein carries an ATP-binding site, ρ protein was treated with the photoaffinity probe 8-azidoadenosine 5′-triphosphate (32) or with the chemical affinity label adenosine 2′,3′-dialdehyde 5′-triphosphate (the product of periodate oxidation of the ribose moiety of ATP) (33) prior to trypsin digestion. The proteolytic fragments were tested for bound affinity label, with somewhat conflicting results. It appears, on the basis of these experiments, that both fragments could contain at least one specific or nonspecific binding site for ATP (unpublished results).

Proteolytic Probing of ρ Conformation. To learn more about the conformational changes induced in ρ protein by the binding (and hydrolysis) of various ligands, we studied the effects of such ligands on the rates of loss of ρ ATPase and intact ρ monomers during proteolysis.

Fig. 4 shows that ρ ATPase activity is lost appreciably more slowly as a consequence of tryptic cleavage when ρ binds ATP[S] than it is in the presence of ATP or ADP at the same concentrations. Gel experiments run in parallel (data not shown) suggest that the cleavage of ρ monomers to fragments f1 and f2 also proceeds more slowly in the presence of ATP[S]. Thus, the binding of this non-hydrolyzable ATP analogue seems to favor a conformational form of ρ that is more resistant to proteolysis than that present during and after ATP hydrolysis. Control experiments (not shown) indicate that, in the absence of trypsin, the ATPase activity of ρ is constant over the entire experimental period.

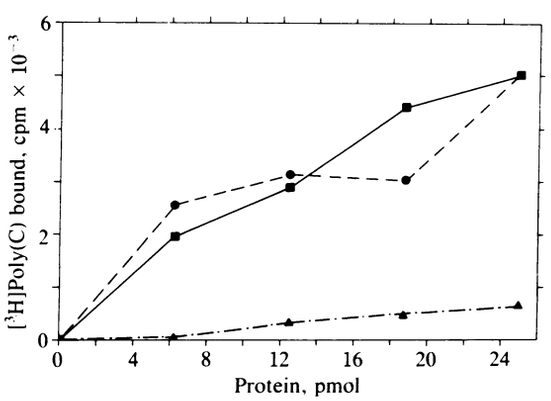

FIG. 3. Nitrocellulose filter assay of the binding of [³H]poly(C) to renatured ρ (—■—), f1 (— —●— —), and f2 (—·—▲—·—). See *Materials and Methods*.

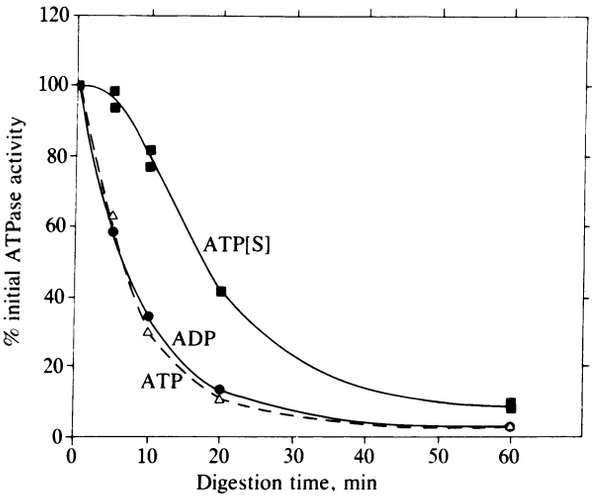

FIG. 4. ATPase activity of ρ as a function of time of trypsin digestion in the presence of ATP and ATP analogs. The original reaction mixture contained 63 μl of TDMK buffer, 17% (vol/vol) glycerol, 15 μg of ρ protein, 7 μg of poly(C), and 1 mM ATP (△), ATP[S] (■), or ADP (●). An initial 10-μl aliquot of the reaction mixture was removed and placed in 10 μl of soybean trypsin inhibitor solution (1 mg/ml). Trypsin solution (7 μl, 1 mg/ml) was then added and the reaction mixture was incubated at 25°C. At the indicated times, 10-μl aliquots were removed from the reaction mixture and placed in 10 μl of soybean trypsin inhibitor solution (1 mg/ml) and kept on ice until the end of the experiment. ATPase measurements were carried out by a modification of the procedure of Lowery and Richardson (7), and the data were corrected for initial dilution by the trypsin solution. In separate experiments, it was shown that the ρ ATPase activity is stable over the course of the experiment in the absence of trypsin (data not shown). Neither the trypsin nor the soybean trypsin inhibitor solutions showed significant levels of ATPase activity.

We have also carried out a number of proteolysis experiments in which we compared the rate of loss of ρ ATPase with the rate of loss of intact ρ monomer. Though the results were quantitatively variable, they show that under our digestion conditions in the presence of RNA and ATP, the rate of loss of ρ ATPase generally exceeded the rate of loss of uncleaved ρ monomer, suggesting that some form of functional cooperativity between the subunits of the ρ hexamer may be required for RNA-dependent ATPase activity.

DISCUSSION

In this study, we have used trypsin to probe the conformations of the monomer subunits of the ρ hexmer in the presence and absence of bound polynucleotides and ATP or ATP analogs. We have shown that when ρ is bound to these cofactors or substrates, which are required for ρ-dependent termination activity, a particular peptide bond (Lys_{283}-Val_{284}) becomes specifically susceptible to cleavage by trypsin. The two major polypeptide fragments that result are discrete and non-overlapping, with a total molecular weight close to the molecular weight of the intact ρ monomer. The fragments, designated f1 and f2, contain the amino-terminal two-thirds and the carboxyl-terminal one-third of the ρ molecule, respectively. Thus, each ρ monomer, when bound to RNA and

ATP within the hexameric structure, seems to exist in a discrete conformation comprising two trypsin-resistant domains connected by a bridge region that is accessible to cleavage at a specific site.

The data show that the most definitive generation of f1 and f2 occurs in the presence of ATP and polynucleotide; in the absence of this substrate and cofactor, cleavage occurs at a variety of other sites as well. The presence of polynucleotide alone greatly enhances the proteolysis of ρ and stimulates the preferred production of a fragment designated f3. In contrast, the presence of ATP alone enhances the resistance of ρ to tryptic digestion. These changes in cleavage patterns suggest that the activation of the ρ NTPase by RNA binding and by substrate binding and cleavage result in one or more changes in the conformation of the ρ monomer. Since activation of ρ NTPase activity by RNA binding followed by ATP hydrolysis seems to be an essential part of the ρ-dependent transcription termination process (8–10), it is tempting to implicate some of these conformational transitions in the termination process.

In an independent study, Engel and Richardson (34) have also observed polynucleotide- and ATP-dependent changes in the rate of tryptic digestion of ρ protein. Many of their results and conclusions concerning the conformational consequences of ligand and substrate binding to ρ are similar to those we present here, though some of their experiments were carried out under different conditions and may reflect somewhat different aspects of ρ structure and subunit interaction.

It has been shown previously that the ρ hexamer can bind a large stretch of RNA (60–90 nucleotides), most likely by wrapping the RNA around the hexameric perimeter (ref. 28 and unpublished results). Conformational transitions of ρ induced by the association and dissociation of RNA, ATP, and possibly RNA polymerase could change the stoichiometry and/or the site size for the binding of RNA around the ρ hexamer. Such ligand-induced changes in the association of ρ with the transcript could provide a mechanical means to couple the process of ATP hydrolysis to conformationally sensitive interactions between ρ and RNA polymerase, resulting ultimately in the dissociation of the RNA·DNA hybrid that is located within the unwound portion of the transcribed DNA. Elsewhere (35, 36; see also refs. 3 and 10) some of us have proposed a two-step mechanism for ρ-dependent transcription termination, based on detailed experiments with the λ promoter P_R-initiated transcript. An ATPase-dependent conformational transition of the sort described here, activated by ρ binding to a specific site on the nascent mRNA, could be centrally implicated in the mechanism whereby ρ brings about detachment of the growing RNA chain from the DNA template and thus induces termination.

In addition to demonstrating a conformational change of this type in ρ, our results can also be used to help map binding sites on the ρ monomer. The f1 fragment of ρ appears to contain at least the major portion of the binding site(s) for poly(C) (and thus presumably that for nascent mRNA). We also attempted (see *Results*) to use affinity probes to localize the ATP binding site(s) of ρ on the f1 and f2 domains, but our findings were not definitive. However, regardless of which domain may be involved in direct ATP binding, it is clear that the rate of production of both fragments is very dependent on the presence or absence of ATP or ATP analogues, implying that the conformations (and/or interactions) of both domains are perturbed by ATP binding and/or hydrolysis. These findings could reflect changes in the relative positions of the domains within the monomer, with little effect on the overall conformation of the hexamer, or they could be due to a change in the exposure of the hinge region (and thus the cleavable bond) as a consequence of the relative movement of whole monomers within the hexamer. Because the hexamer appears to be the active form of ρ in termination (4, 37), we would expect that important conformational changes should affect the interactions of the hexamer, as a whole, with RNA.

Finally, we would like to point out that the domain structure of the monomers of the activated hexamer of ρ that emerges from these studies and from those of Engel and Richardson (34) may be characteristic of a whole class of related proteins. For example, the single-stranded-DNA-dependent ATPase dnaB protein involved in DNA replication in *E. coli* has also been shown, by tryptic cleavage, to consist of two structural domains (38); like ρ, the dnaB protein is a hexamer of six identical subunits (39, 40). Together, these results are consistent with the hypothesis that the regulatory and catalytic portions of many nucleic acid binding proteins initially evolved separately and then were brought together as functionally linked domains by gene fusion. Subsequent assembly of these two-domain proteins into oligomeric species subject to conformational rearrangement added more layers of regulatory sophistication. It will be interesting to see whether this pattern shows up in other proteins involved in the regulation of gene expression at various levels.

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