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Structure of ρ factor: An RNA-binding domain and a separate region with strong similarity to proven ATP-binding domains

(transcription termination factor/chemical modification/domain structure/protein cleavage/ATPase)

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ABSTRACT **The domain structure of ρ protein, a transcription termination factor of *Escherichia coli*, was analyzed by oligonucleotide site-directed mutagenesis and chemical modification methods. The single cysteine at position 202, previously thought to be essential for ρ function, was changed to serine or to glycine with no detectable effects on the protein's hexameric structure, RNA-binding ability, or ATPase, helicase, and transcription termination activities. A 151-residue amino-terminal fragment (N1), generated by hydroxylamine cleavage, and its complementary carboxyl-terminal fragment of 268 amino acids (N2) were extracted from NaDodSO$_4$/polyacrylamide gels and renatured. The N1 fragment binds poly(C) and mRNA corresponding to the ρ-dependent terminator sequence *trp t'*, but not RNA unrecognized by ρ; hence, this small renaturable domain retains not only the binding ability but also the specificity of the native protein. Uncleaved ρ renatures to regain its RNA-dependent ATPase activity, but neither N1 nor N2 exhibits any detectable ATP hydrolysis. Similarly, the two fragments, isolated separately but renatured together, are unable to hydrolyze ATP. Sequence homology to the α subunit of the *E. coli* F$_1$ membrane ATPase, and to consensus elements of other nucleotide-binding proteins, strongly suggests a structural domain for ATP binding that begins after amino acid 164. The implications of discrete domains for RNA and nucleotide binding are discussed in the context of requirements for specific interactions between RNA-binding and ATP-hydrolysis sites during transcription termination.**

ρ protein of *Escherichia coli* is a transcription termination factor that catalyzes 3′ endpoint formation and release of mRNA molecules from DNA templates (1–3). This essential protein has identical subunits of 419 amino acids (4, 5) and exists as a hexamer in solution (5, 6). ρ binds to both single-stranded RNA and single-stranded DNA (7, 8); its highest known affinity is for synthetic polycytidylate [poly(C)] (9). When activated by binding to RNA, ρ has the ability to hydrolyze ATP in the absence of other transcriptional components (9, 10); this RNA-dependent ATPase activity is also necessary for transcription termination (11).

We recently demonstrated that the RNA-dependent ATPase activity is crucial as well for the RNA·DNA helicase activity of ρ (12). This activity most likely serves the observed transcriptional function of releasing completed mRNA from the DNA template (13, 14). Our understanding of the functional mechanisms by which ρ catalyzes transcription termination is thus becoming clearer, yet our knowledge of the structural determinants necessary for RNA binding and ATP hydrolysis, and the interactions between them that are required for termination, is still very limited. ρ's RNA-dependent ATPase activity (essential for termination) can be inactivated by reagents that react with free sulfhydryl groups (15), and one attractive possibility for RNA recognition proposed that a transient covalent intermediate (termed a Michael adduct) might form between the sole cysteine at position 202 in each ρ subunit (4) and regularly spaced cytosine bases in the RNA substrate to "nucleate" ρ binding (1). In this paper we demonstrate that Cys-202 is completely dispensable for ρ function, thereby unequivocally ruling out the Michael adduct hypothesis for recognition. We report the results of attempts to define more precisely the RNA-binding and ATPase regions within the ρ protein and discuss how the RNA-binding domain may interact with an adjacent ATP-binding domain to catalyze RNA-dependent ATP hydrolysis and transcription termination.

MATERIALS AND METHODS

Oligonucleotide Site-Directed Mutagenesis. The ρ gene was removed from plasmid p39-AS (16) by cleavage with restriction endonucleases *Bcl* I and *Hin*dIII and subcloned into the *Bam*HI and *Hin*dIII sites of pUC118 (a phage M13-derived vector from J. Vieira, Waksman Institute, Rutgers, NJ). The resulting plasmid, pAJD1, was used to transform competent *E. coli* strain RZ1032 *ung, dut* (17). Single-stranded plasmid DNA containing uracil was generated by infection with the helper phage R408, from Stratagene (San Diego, CA). Synthetic oligonucleotides d(CCGGATAGTGTGCTGATG) (for the cysteine-to-serine mutation) and d(CCGGATGGTGTG-CTGATG) (for the cysteine-to-glycine mutation) were prepared with a DNA synthesizer (Applied Biosystems, Foster City, CA). The two-primer method (18) was used for mutagenesis *in vitro*, and the resulting double-stranded DNA molecule was transformed into *E. coli* strain BMH71-18 *mutL* (19), which is defective for mismatch repair, to improve the efficiency of mutagenesis. Colonies were probed by colony hybridization (20) with the same oligonucleotide used for mutagenesis but now ^{32}P-labeled at the 5′ end. Single-stranded DNA from positive colonies was used as template for ^{35}S/dideoxy sequencing (21, 22).

Overproduction of Proteins. The mutated ρ genes were removed from pAJD1 on an *Eco*RV restriction fragment of ≈1800 base pairs and ligated into the *Eco*RV restriction sites of p39-AS. These plasmids were transformed into *E. coli* strain AR120-A6, a *dam*::Tn*9* derivative of AR120 (16). Nalidixic acid induction (16) yielded high levels of mutant ρ proteins that were partially purified by centrifugation on sucrose density gradients. Approximately 1 g of cells was broken in a French pressure cell press and stored frozen; extracts were thawed, resuspended to 2 ml in buffer A (23), and microcentrifuged for 15 min at 4°C. The supernatant was diluted to 1.5 ml with the same buffer and 0.1 ml was loaded onto each 5-ml sucrose gradient (5–20% sucrose). The gradients were run in a Beckman SW50.1 rotor to $\omega^2 t = 6 \times$

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Abbreviations: NTCB, 2-nitro-5-thiocyanobenzoic acid; *p*HMB, *p*-hydroxymercuribenzoate; MalNEt, *N*-ethylmaleimide.

10^{11} at 4°C. Nine fractions were collected from the bottom of the gradient and analyzed by 0.1% $NaDodSO_4$/10% polyacrylamide gel electrophoresis, and the peak ρ fraction (second from bottom) was chosen for subsequent analyses.

Protein Analysis *in Vitro*. For RNA-binding studies poly(C) was either 5′- or 3′-end-labeled, purified by electrophoresis in 7 M urea/10% polyacrylamide gel, eluted by soaking in 0.3 M sodium acetate/0.1 mM EDTA, filtered, precipitated, and resuspended in 50–75 μl of water. Binding of ρ protein to ^{32}P-labeled poly(C) was carried out in ATPase assay buffer plus 0.5 mM ATP, 0.9 nCi (33.3 Bq) of ^{32}P-labeled poly(C) [≈100 nM in nucleotides, or 1 nM poly(C) with a range of lengths averaging about 100 nucleotides], and 0–75 nM ρ protein in a total reaction volume of 50 μl. The mixture was incubated at 37°C for 20 min, followed by filtration of 45 μl through 13-mm-diameter nitrocellulose filters (Schleicher & Schuell). The filters were washed twice with 45 μl of ATPase buffer, dried briefly, and subjected to scintillation counting to measure the RNA bound to ρ. Synthesis of the ρ-dependent termination sequence *trp t′*, and of its complementary mRNA *in vitro* by use of SP6 RNA polymerase, has been described (12).

Chemical Modification of ρ Protein. ρ protein was cleaved at Cys-202 with 2-nitro-5-thiocyanobenzoic acid (NTCB; Fig. 1) (24), by adding $NaDodSO_4$ (final concentration 0.1%) to 100 μl of ρ from gradient fraction 2 plus 10 μl of 37 mM NTCB (in ethanol) and incubating at 37°C for 30 min. Then 100 μl of 1 M acetic acid was added, followed by 1 ml of ice-cold acetone. The protein was precipitated by microcentrifugation for 5 min at 4°C. The pellet was washed twice with 0.5 ml of cold acetone, dried, and resuspended in 20 μl of 1% $NaDodSO_4$. One hundred microliters of 0.1 M sodium borate (pH 9.6) was added and the reaction mixture was incubated for 14–16 hr. Products were analyzed by 0.1% $NaDodSO_4$/10% polyacrylamide gel electrophoresis. Uncleaved ρ polypeptide chains and the cleavage fragments were eluted from polyacrylamide gels and renatured according to Hager and Burgess (25). Cleavage at Asn-151 by hydroxylamine was performed in a reaction mixture containing 70 nM ρ, 1.7 M NH_2OH (dissolved in 2 M Tris·HCl/2 M LiOH), and 0.2% $NaDodSO_4$. Following incubation at 45°C for 3 hr, acetic acid was added to 0.5 M. The protein was precipitated using ice-cold acetone (as for the NTCB cleavage), dried, and resuspended in 50 μl of 0.8% $NaDodSO_4$. Fragments were eluted and renatured in the same manner as for NTCB cleavage. Modification of ρ with *N*-ethylmaleimide (MalNEt) was achieved in reaction mixtures consisting of 70 nM ρ from gradient fraction 2, ATPase buffer, and 100 mM MalNEt. Cys-202 in ρ was modified by adding *p*-hydroxymercuribenzoate (*p*HMB, dissolved in 0.1 M NaOH to 50 mM) to 70 nM ρ from sucrose gradient fraction 2, for a final *p*HMB concentration of 7.5 mM in ATPase buffer.

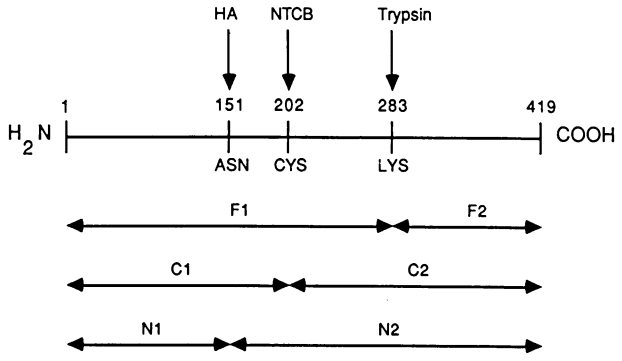

FIG. 1. Linear diagram of ρ protein. Cleavage sites for hydroxylamine (HA), NTCB, and trypsin are indicated. A comparison of the fragments generated by each type of cleavage event appears below the linear protein.

RESULTS

Cys-202 Is Completely Dispensable for ρ Function. Site-directed mutagenesis was used to alter the codon for Cys-202 to code for either glycine or serine (the resulting proteins will be referred to as Cys→Gly and Cys→Ser); desired changes were identified by colony hybridization and verified by dideoxy sequence analysis. The altered ρ genes were cloned in plasmid p39-AS, where the expression of ρ is under the control of the λ P_L promoter. Mutant ρ proteins were overproduced by nalidixic acid induction (16).

Overproduced Cys→Gly and Cys→Ser proteins were partially purified by sucrose density gradient centrifugation and were found to sediment coincident with wild-type ρ within the resolution of the gradient, indicating that they also form hexamers correctly; we estimate that a 10% change would have been detectable. The identity of the mutant proteins was checked using the cysteine-specific chemical cleavage reaction by NTCB, which results in two fragments of about 22 and 24 kDa (Fig. 2*B*, lane 1). The absence of cleavage for the identically treated Cys→Gly and Cys→Ser proteins confirmed that the cysteine residues had been altered (Fig. 2*B*, lanes 2 and 3).

We further characterized the partially purified mutant ρ factors *in vitro* by analyzing (*i*) the RNA-dependent ATPase activity, (*ii*) the RNA·DNA helicase activity, and (*iii*) the ability to terminate transcription at the ρ-dependent terminator *trp t′*. Fig. 2*A* shows the results of ATPase assays using poly(C) as the RNA activator. The behavior of both Cys→Gly and Cys→Ser proteins did not deviate significantly from that of the wild-type control, indicating that the ATPase activity of ρ does not require Cys-202. A more detailed kinetic analysis (unpublished data) gave K_m values of 17.9, 15.6, and 14.6 μM for wild-type, Cys→Ser, and Cys→Gly proteins, respectively, with corresponding V_{max} values of 33.1, 37.3, and 24.3 pmol/min. Substitution of *trp t′* RNA for poly(C) also did not reveal any significant differences, nor did the RNA·DNA helicase and transcription termination abilities of the mutant ρ factors differ from those of wild-type ρ (data not shown). Thus Cys-202 is not essential, contrary to our assumption based upon the inactivation of ρ by sulfhydryl-modifying reagents (15), and the binding of ρ to RNA by nucleation at cytosine bases via Michael adduct formation as proposed by Platt (1) does not make a significant contribution to ρ function.

An Amino-Terminal Fragment of 151 Amino Acids Binds RNA. ρ protein has a single asparagine–glycine bond, at position 151–152; this bond can be cleaved by hydroxylamine (26), resulting in a 151 amino acid fragment (N1) derived from the amino terminus of the protein and a 268 amino acid fragment (N2) from the carboxyl terminus (Fig. 3*A*). An amino-terminal segment of 283 amino acids (called F1) can bind poly(C) as well as the wild-type molecule (27), and hydroxylamine cleavage cuts this F1 RNA-binding peptide roughly in half. To determine whether RNA binding was retained by either of our hydroxylamine fragments, N1 and N2 were eluted from a $NaDodSO_4$/polyacrylamide gel and renatured (25). Each fragment was renatured both individually and in combination with its complementary fragment. Both intact ρ (never exposed to hydroxylamine) and uncleaved ρ (that which remained due to incomplete cleavage) were extracted from the same gel as the cleaved samples and were renatured as positive controls. RNA-binding ability and ATPase activity were recovered with good efficiency for both the intact and uncleaved ρ proteins, as observed previously (25, 27).

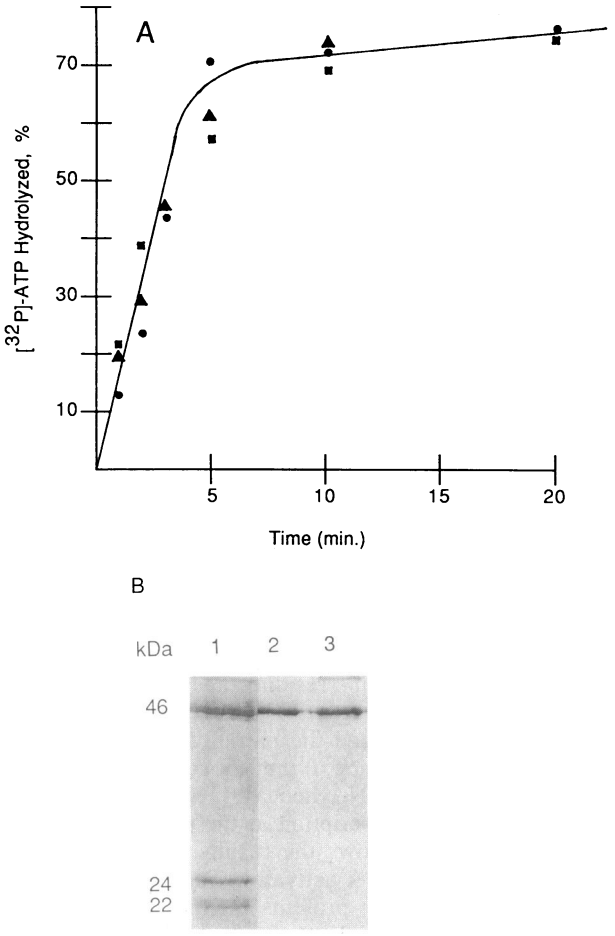

FIG. 2. Characterization of Cys→Gly and Cys→Ser mutant ρ proteins. (*A*) ATPase activity of wild-type ρ (●) and the Cys→Gly (■) and Cys→Ser (▲) mutants. The ATPase activity of ρ was evaluated in 10-μl reaction mixtures containing 50 mM KCl, 20 mM Tris acetate (pH 7.9), 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.4 mM magnesium acetate, 5 μM poly(C) (average length, 100 nucleotides), 10 μM ATP, 45 nCi of [γ-^{32}P]ATP (specific activity ≈3000 Ci/mmol, from Amersham), and sucrose gradient-purified ρ (1 nM). After incubation at 37°C for various times, 1-μl samples were spotted onto polyethyleneimine-cellulose plates. ATP and $^{32}P_i$ were separated by chromatography in 0.75 M KH_2PO_4, located by autoradiography, and cut out for scintillation counting. (*B*) NTCB cleavage fragments for sucrose density gradient-purified wild-type ρ (lane 1), Cys→Gly ρ (lane 2), and Cys→Ser ρ (lane 3). Alterations at Cys-202 are verified by the lack of cleavage by NTCB. Positions of intact ρ (46 kDa), the amino-terminal C1 fragment (22 kDa), and the carboxyl-terminal C2 fragment (24 kDa) are indicated.

The ability of N1 and N2 to bind to RNA was determined in filter binding assays with poly(C) as the substrate and with a natural substrate for ρ binding, *trp t'* mRNA. A negative control was included by using mRNA from the strand opposite the *trp t'* region. Eluted intact ρ and uncleaved ρ from the renaturation step were again used as the controls for renaturation. Fig. 3*B* shows that renatured N1 binds to poly(C) in a similar manner to intact renatured ρ, whereas fragment N2 shows no significant RNA-binding ability. When N1 and N2 were renatured together, or mixed after individual renaturation, RNA-binding ability was the same as with N1 alone or uncleaved ρ. N1 also bound *trp t'* mRNA, though less well than poly(C), but did not bind an mRNA complementary to the *trp t'* sequence (data not shown). The specificity for binding of RNA by N1 is thus similar to that for intact ρ protein, and significant interactions with RNA simply due to the basic character of N1 can be ruled out. All the RNA-binding ability and specificity of ρ protein therefore appears to reside in the N1 fragment. The renatured N1 and N2 fragments were also assayed individually for ATPase activity, but none was detected. Fragments that had been renatured together or mixed together after separate renaturation likewise showed no RNA-dependent ATP hydrolysis.

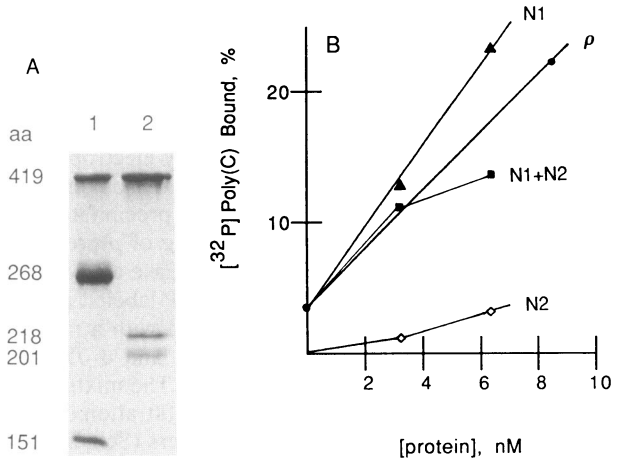

FIG. 3. RNA binding of ρ fragments generated by chemical cleavage. (*A*) Cleavage of ρ with hydroxylamine (lane 1) and NTCB (lane 2). The fragments were eluted from 0.1% $NaDodSO_4$/10% polyacrylamide gels and renatured (26) for analysis *in vitro*. The lengths are indicated in number of amino acids (aa). (*B*) Nitrocellulose filter assay of the binding of ^{32}P-labeled poly(C) to renatured ρ and the hydroxylamine-cleavage fragments of ρ, N1 and N2, as described in *Materials and Methods*. RNA bound (%) is plotted as a function of protein concentration.

Cleavage of ρ with NTCB (Fig. 1) results in an amino-terminal fragment of 201 amino acids (C1) and a carboxyl-terminal fragment of 218 amino acids (C2). The ATPase and RNA-binding capabilities of each of these fragments were tested just as for N1 and N2. Neither fragment alone or the two renatured together possessed any ATPase activity. As expected, based on the results for the binding of RNA to F1 and N1, C1 was also able to bind RNA, as is the mixture of C1 and C2, whereas C2 alone showed no RNA binding ability (data not shown).

A Domain for ATP Binding Is Suggested by Sequence Homology with Other ATP-Binding Proteins. A computer-generated scan of the GenBank protein database showed strong sequence homology between ρ and several ATPases (28); the best overall homology to ρ was found in the α subunit of the *E. coli* F_1-ATPase. Our direct comparisons, using the University of Wisconsin Genetics Computer Group software package with the Bestfit and Gap programs, revealed that ρ is 41% similar (identities plus conservative changes) to the F_1-ATPase α subunit and has specific regions of near identity that are characteristic of ATP-binding domains. Though the similarity of ρ to the β subunit is also 41%, stretches of identical sequence are rarer. We have characterized ρ as an RNA·DNA helicase (12), but it has only 35% similarity to the DnaB helicase of *E. coli* and has no long stretches of identical sequence. Whether the greater similarity of ρ to the F_1 α subunit reflects an evolutionary rather than a functional relationship remains to be determined.

For two related ATP-binding proteins, adenylate kinase (whose crystal structure is known) and F_1 β subunit, five parallel β-strands (in the order 2–3–1–4–5) probably form a hydrophobic pocket for the ATP molecule (29, 30). Fig. 4 displays the sequence of ρ protein aligned with the α and β subunits of the F_1-ATPase and with adenylate kinase, and the β-strand predictions agree with those predicted for ρ (4).

Immediately adjacent to the β1 region (where ρ and the F_1 α subunit are virtually identical) is the A consensus sequence

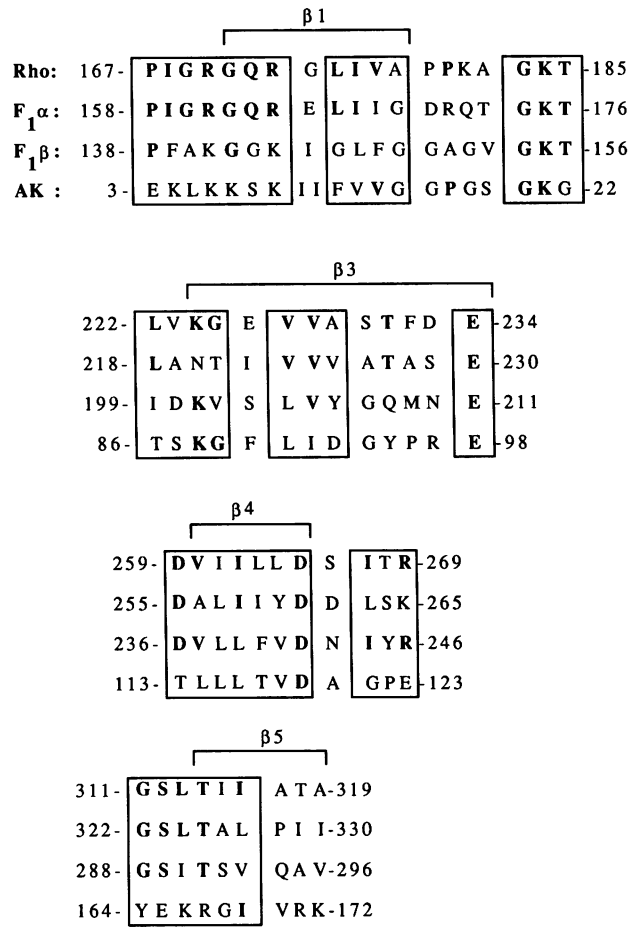

FIG. 4. Sequence and β-structure alignment of ρ with three similar ATP-binding proteins ($F_1\alpha$ and $F_1\beta$, α and β subunits of bacterial F_1-ATPase; AK, adenylate kinase). Parallel β-stranded regions β1, β3, β4, and β5 are adjacent and form part of the hydrophobic pocket for ATP in the three-dimensional structure of adenylate kinase and that proposed for F_1 β subunit (29, 30). The alignment between the α and β subunits of the bacterial F_1-ATPase (for β1, β3, β4, and β5) and adenylate kinase (for β1 and β4) are essentially as determined by Walker *et al.* (31) with matrix matching programs; adenylate kinase alignment with β3 and β5 is based on its tertiary structure (29, 30). We differ only in β4 of adenylate kinase, which is shifted by one residue to maintain alignment of its critical Asp-119 (see text) with those of the other three proteins. Sequence identity is indicated by boldface type; boxes include conservative substitutions except for a few deviations. Amino acids are represented by standard one-letter symbols.

of Walker *et al.* (31), Gly-Xaa$_4$-Gly-Lys-Thr, seen in many nucleotide-binding proteins and highly conserved among all four proteins shown. This region forms a flexible loop that may interact with bound nucleotides, and the lysine following this loop is believed to contact the α phosphate (30).

A second region of strong homology is the B consensus sequence (31) that comprises β4: a very hydrophobic region that forms a β-sheet at the back of the nucleotide pocket is followed by a highly conserved aspartic residue that has been implicated in interactions with Mg^{2+} of Mg-ATP (30). The agreement between ρ and the F_1 ATPase subunits in having β3 and β5 homologies as well, at equivalent positions, supports a proposal that the tertiary structures are likely to be similar. A final argument that Fig. 4 reflects part of the structure of the nucleotide-binding site of ρ, rather than general characteristics of ATPases, is provided by the demonstration, by Garboczi *et al.* (32), that a 50-residue peptide corresponding to amino acids 135–184 of the F_1 β subunit can bind ATP (see *Discussion*). The overall similarity of ρ to these elements, which are known to be involved in nucleotide binding, strongly suggests tertiary-structure similarity to F_1 α and β subunits and adenylate kinase, and that ρ's binding site comprises a 180–200 amino acid domain beginning beyond residue 150.

DISCUSSION

An understanding of the function of bacterial transcription termination factor ρ requires a knowledge of how ρ recognizes and binds to its RNA target site, how this leads to ATP hydrolysis, and what interactions occur with the elongating transcription complex to catalyze transcription termination. We previously showed that ρ can unwind an RNA·DNA duplex similar to that present in the transcription "bubble," by a mechanism (still unknown) that utilizes ATP hydrolysis (12). Our current results address structural questions about the protein regions involved in the first stage of ρ activation, RNA binding and recognition, and suggest that ρ factor has discrete functional domains for RNA binding and ATP binding. The RNA-binding domain must be contained within the first 151 amino acids (fragment N1), as shown experimentally; the N2 fragment includes a region of 180 amino acids with some striking sequence homologies to the α subunit of bacterial F_1-ATPase. This region of ρ includes several conserved elements found in many nucleotide-binding proteins and, by comparison with those whose structure is known, provides a basis for a testable prediction of the structure of the ATP-binding domain of ρ.

The first 283 amino acids of the protein were previously shown to bind RNA (27), possibly by using the sole cysteine at position 202 to "nucleate" ρ binding by formation of a transient covalent Michael adduct at cytosine bases in ρ-dependent terminator regions (1). This hypothesis was consistent with observations that ρ could be inactivated by the sulfhydryl-specific reagents MalNEt and *p*HMB (15) and that the spacing of cytosine residues in several ρ-dependent terminators (12 nucleotides apart) corresponds to the approximate size necessary for interaction with the unique cysteine on each of the six ρ subunits (5). This idea was further supported by the very strong preference of ρ for binding to poly(C).

We made oligonucleotide site-directed mutations in the ρ gene that altered Cys-202 and were surprised to find that replacement of this residue with either glycine or serine did not significantly affect any of the various activities of ρ factor that we tested. Thus Cys-202, once thought essential for ρ function, is completely dispensable. In other proteins, sulfhydryls considered essential based on the results of chemical modification have similarly been shown to be unimportant for function (33). This interpretive error appears to be due to (*i*) the alkylation of residues other than cysteine by MalNEt and (*ii*) steric effects by the bulky substituent introduced by *p*HMB modification of Cys-202 rather than due to a requirement for the cysteine *per se*.

The location of the RNA-binding domain was further delineated by chemical cleavage with NTCB (at Cys-202) and hydroxylamine (at Asn-151) to yield fragments smaller than the 283 amino acid F1 binding segment defined by Bear *et al.* (27). These were extracted and renatured from NaDodSO$_4$/polyacrylamide gels along with intact ρ. Both the 201- and the 151-residue amino-terminal fragments can bind to poly(C) in a similar manner to intact ρ factor. N1 also retains specificity, since it can bind to its natural substrate, *trp t'* mRNA, but not to another RNA of similar size. Thus, the ability of ρ to bind specific RNAs can be attributed entirely to its first 151 amino acids (or less), and the lack of significant interaction between RNA and the N2 fragment (Fig. 3*B*) rules out significant contributions to RNA binding outside of N1. The possibility of minor interactions cannot be

eliminated, and one model has suggested that ρ has two kinds of nucleic acid-binding sites (8) and that activation of ATPase activity requires contact between RNA and ρ at both sites.

The N2 domain of ρ shows a striking degree of sequence homology with the α subunit of F_1-ATPase and shares common consensus elements with the β subunit of F_1-ATPase, adenylate kinase, and many other nucleotide-binding proteins (Fig. 4). Crystallographic studies and tertiary-structure predictions indicate that in adenylate kinase and F_1 β subunit, ATP binds within a hydrophobic pocket formed by five parallel β-strands (29, 30).

The β1 strand of ρ and several residues immediately following β1 conform to the A consensus sequence described by Walker *et al.* (31). This consensus, Gly-Xaa$_4$-Gly-Lys-Thr, is in perfect agreement with F_1 α and β subunits; adenylate kinase lacks the final threonine, and in ρ the initial glycine is an alanine [as is also seen in the UvrD protein and elongation factor G (34)]. This region can form a flexible loop, and crystallographic data suggest that in adenylate kinase the nucleotide-binding domain resides near this loop (30); a similar tertiary structure for the nucleotide-binding site of F_1 β subunit appears likely (29, 30). Further conserved features in this region are the basic residues of ρ at positions 170 and 173, and at Lys-184 (in adenylate kinase this lysine is thought to interact with the α phosphate of ATP). Pro-180 is equivalent to Pro-17 in adenylate kinase, which is believed to stabilize the glycine-rich flexible loop (30). Furthermore, Fry *et al.* (30) proposed that the replacement of this proline by arginine and alanine in F_1 α and β subunits, respectively, may account for reduced effectiveness of the hydrolytic activity. Evidence that β1 and the following region participate directly in ATP binding comes from the demonstration that a synthetic peptide corresponding to amino acids 135–184 of F_1 β subunit (of mitochondrial ATP synthase, nearly identical to the bacterial enzyme in this region) binds ATP, probably most strongly to the phosphate ester region, and upon doing so actually precipitates from solution (32).

A second highly conserved region for nucleotide-binding domains occurs within the fourth β-strand (see *Results* text and Fig. 4). The existence of additional sequence homology to β3 and β5 at corresponding positions of the ρ polypeptide chain further supports the idea that ρ probably has a nucleotide-binding pocket with β-structure similar to adenylate kinase and the F_1-ATPase subunits. Less likely structures may be derived from an alternative consensus sequence, Gly-Xaa-Gly-Xaa-Xaa-Gly-Xaa$_n$-Lys, that also appears in ρ. In instances where this is seen, it is proposed as part of a $\beta\alpha\beta$ motif (n = 8–15) (35) or to lie between two β-strands (n = 16–28) (36). The former possibility can be ruled out for ρ because Pro-179 and Pro-180 would fall in the center of the α-helix; the latter is unlikely because of the large distance to the lysine (where n = 9 for ρ).

Since the ATPase activity of ρ is RNA-dependent, and since attempts to recover ATPase activity from renatured N1 and N2 fragments were not successful, we propose a model for ρ protein where N1 plus RNA must interact with a separate ATP-binding domain to facilitate hydrolysis in contacts that are prevented by *p*HMB modification and unattainable after hydroxylamine cleavage. Although N1 was generated chemically, it includes a functional RNA-binding domain. A similarly sized amino-terminal domain (124 amino acids) can be obtained by hydroxylamine cleavage of ribosomal protein S4 (37), and analysis of such relatively small RNA-binding fragments should facilitate our understanding of requirements for RNA–protein recognition.

Our study of structure–function relationships within ρ protein suggests a discrete domain for RNA binding contained within the first 151 amino acids of the protein. This region is separate from the proposed ATP-binding domain, since the sequence homologies to nucleotide-binding proteins begin after amino acid 164. We infer that the domains for RNA binding and ATP binding comprise separate regions of the protein but that interaction between them is necessary for ATP hydrolysis.

Note Added in Proof. S. E. Seifried, Y. Wang, and P. von Hippel (personal communication) have also found that modification of Cys-202 does not affect ρ function.

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