Ribonucleic Acid Synthesis Termination Protein ρ Function: Effects of Conditions That Destabilize Ribonucleic Acid Secondary Structure†

John P. Richardson* and Martha R. Macy

ABSTRACT: The dependence of the rate of adenosine 5′-triphosphate (ATP) hydrolysis catalyzed by ribonucleic acid (RNA) synthesis termination protein ρ from *Escherichia coli* with T7 RNA as cofactor is used to probe the nature of the interaction between ρ and RNA. In general, reaction conditions that destabilize the secondary structure of the RNA enhance its cofactor activity. This is indicated by the effects of $MgCl_2$ concentration, spermidine, temperature, dimethyl sulfoxide, and pretreatment of the RNA with formaldehyde. These results suggest that a functional interaction between ρ and RNA depends either on the presence of a sufficiently large single-stranded region in the RNA or on the ability of ρ to unwind double helices in the RNA. It is also shown that changes in reaction conditions that increase RNA secondary structure and decrease the ρ protein adenosine triphosphate phosphohydrolase (ρATPase) activity with isolated T7 RNA also decrease the stringency of ρ action in RNA synthesis termination. On the other hand, monovalent salts decrease ρATPase activity with isolated T7 RNA and binding of ρ to T7 RNA independently of the $MgCl_2$ concentration and thus the relative stability of the RNA secondary structure.

RNA[1] synthesis catalyzed by RNA polymerase from *Escherichia coli* is terminated at specific sites on DNA templates by action of ρ protein (Roberts, 1969, 1976; Adhya & Gottesman, 1978). This process depends on the presence of nucleoside triphosphates that can be hydrolyzed to nucleoside diphosphates and P_i by ρ action (Howard & deCrombrugghe, 1976; Galluppi et al., 1976). An essential function of ρ in termination is to cause the release of RNA chains from transcription complexes containing RNA polymerase and DNA (Roberts, 1969), and this release reaction is driven by the hydrolysis of nucleoside triphosphates (Richardson & Conaway, 1980). However, the hydrolysis of NTP's by ρ action need not be coupled to RNA synthesis termination, since ρ catalyzes the hydrolysis reaction in the presence of RNA alone (Lowery-Goldhammer & Richardson, 1974). The ability to measure the NTPase activity of ρ with isolated RNA as a cofactor provides a convenient technique for studying the nature of the interactions between ρ and RNA that are related to the function of ρ in termination.

The NTPase activity of ρ has an absolute requirement for RNA. However, this requirement is not satisfied by all RNAs equally. Some RNA molecules, such as poly(C) and other pyrimidine-rich polymers containing cytidylate residues, are at least 100-fold more effective in activating ρNTPase than are certain other RNA molecules such as poly(G) or tRNA (Lowery-Goldhammer & Richardson, 1974; Lowery & Richardson, 1977b; Adhya & Gottesman, 1978). Apparently, the structure of the RNA is very important, and even poly(C) becomes ineffective as an activator when it is complexed with poly(I).

Isolated T7 RNA prepared by transcription of T7 DNA in vitro has a cofactor activity that is intermediate between the activity of poly(C) and those of poly(G) and tRNA (Lowery-Goldhammer & Richardson, 1974). However, since the cofactor activity of the RNA depends on its structure and since the structure of T7 RNA is sensitive to solvent and temperature conditions, ρNTPase activity with isolated T7 RNA could depend very strongly on the reaction conditions, and, as a consequence, the conditions could also affect the specificity of ρ function in termination.

It has been shown already by Adhya et al. (1979) that ρ function is enhanced when RNA is synthesized with nucleotides that decrease the stability of the secondary structure and is reduced when RNA is synthesized with nucleotides that increase the secondary structure. In this paper, we present results on the influence of reaction conditions that destabilize RNA secondary structure on ρATPase activity with isolated T7 RNA and on the stringency of ρ function in terminating transcription of T7 DNA. The results confirm and extend conclusions of Adhya et al. (1979) and show, as well, that the specificity of ρ function in vitro can be altered significantly by manipulating the condition used for RNA synthesis with normal substrates.

Materials and Methods

Chemicals and Biochemicals. ATP, GTP, CTP, and UTP were purchased from Boehringer-Mannheim Corp. [^{3}H]UTP and $^{32}P_i$ were obtained from ICN Pharmaceuticals Inc., and [γ-^{32}P]ATP was prepared as described previously (Lowery & Richardson, 1977a). *E. coli* total tRNA was from General Biochemicals, and pancreatic deoxyribonuclease (code DPFF) was purchased from Worthington Biochemical Corp. Sephadex G-50 was from Pharmacia, and DEAE-cellulose was DE52 from Whatman. Rifampicin (rifampin) was a gift from the Ciba-Geigy Corp. Acrylamide, bis(acrylamide), and sodium dodecyl sulfate were obtained from Bio-Rad Laboratories. Urea was "ultra-pure" from Schwarz/Mann. All other chemicals were reagent grade. *E. coli* MRE 600 cells, grown to late log phase in an enriched medium, were purchased from Grain Processing Corp., Muscatine, IA.

† From the Department of Chemistry and the Program in Molecular, Cellular and Developmental Biology, Indiana University, Bloomington, Indiana 47405. *Received August 12, 1980.* This work was supported by a research grant from the National Institutes of Health (AI 10142).

[1] Abbreviations used: RNA, ribonucleic acid, DNA, deoxyribonucleic acid; NTP's, nucleoside triphosphates; ρNTPase, ρ protein nucleoside triphosphate phosphohydrolase; ρATPase, ρ protein adenosine triphosphate phosphohydrolase; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; UTP, uridine 5′-triphosphate; ITP, inosine 5′-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; poly(C), poly(cytidylic acid); poly(G), poly(guanylic acid); poly(I), poly(inosinic acid).

0006-2960/81/0420-1133$01.25/0

RNA Polymerase and ρ and T7 DNA. RNA polymerase purified from *E. coli* MRE 600 by the method of Burgess & Jendrisak (1975) was purified further by chromatography on DEAE-cellulose (Chamberlin & Berg, 1962). ρ protein was isolated from *E. coli* MRE 600 by a procedure of Finger & Richardson (1981). T7 DNA was prepared by phenol extraction of purified bacteriophage T7 (Thomas & Abelson, 1966).

T7 RNA. RNA was synthesized from T7 DNA in vitro in a reaction mixture that contained 40 μg of T7 DNA and 20 μg of *E. coli* MRE 600 RNA polymerase in 1 mL of a solution containing 1.5 mM ATP, 1 mM GTP, 0.5 mM each of UTP and CTP, 0.04 M Tris-HCl buffer (pH 8.0), 0.1 M KCl, 12 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 mM EDTA. After incubation for 100 min at 37 °C, 75 μg of pancreatic DNase (RNase-free) was added and the incubation continued for 5 min at 22 °C before adding 0.1 mL of 0.5 M EDTA and 0.02 mL of 10% sodium dodecyl sulfate. Proteins were removed by extracting the solution 2 times with water-saturated phenol. After the second phenol extraction, the solution was extracted 3 times with 2-butanol to remove phenol and to reduce the volume of the aqueous phase to 0.4 mL. This solution was filtered through a 1 × 29 cm column of Sephadex G-50 in a solution containing 0.04 M Tris-HCl buffer (pH 8.0) and 0.05 M KCl. The RNA eluted in the void volume, and the DNA fragments and unincorporated nucleotides were retarded on this column. The peak RNA fractions were pooled and stored frozen. The concentration of RNA is estimated by assuming $\epsilon_{260}^{1\%} = 25$. For preparation of T7 [^{3}H]RNA, the reaction mixture contained 0.25 mM [^{3}H]UTP (100 Ci/mol).

Assay of ρATPase. Hydrolysis of ATP to ADP and P_i was determined by measuring the release of $^{32}P_i$ from [γ-^{32}P]ATP as described by Lowery & Richardson (1977a). The standard reaction mixture contained 0.04 M Tris-HCl buffer (pH 8.0), 0.05 M KCl, 1 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (300–600 cpm/nmol), 10^{-4} M dithiothreitol, 10^{-4} M EDTA, 20 μg of bovine serum albumin, 0.5 μg of T7 RNA, and 0.5 μg of ρ in 0.1 mL. The standard incubation was 30 min at 37 °C. The RNA is the limiting component in this reaction mixture; thus, the activity is very sensitive to changes in the cofactor activity of the RNA.

Assays for Transcription Termination and Nucleotide Incorporation Assay. Standard RNA polymerase reaction mixtures used to measure the effect of ρ on the incorporation of nucleotides into RNA were prepared by the following procedure: 0.84 μg of RNA polymerase and 4 μg of T7 DNA were preincubated for 10 min at 37 °C in 45 μL of a solution containing 0.04 M Tris-HCl buffer (pH 8.0), 0.05 M KCl, 5 mM $MgCl_2$, 0.2 mM dithiothreitol, and 0.2 mM EDTA. After addition of 0.6 μg of ρ in 10 μL of 0.04 M Tris-HCl, 0.05 M KCl, 5 mM $MgCl_2$, and 0.1 mM dithiothreitol, RNA synthesis was initiated by adding 0.1 μmol each of ATP, GTP, and CTP, 0.02 μmol of [^{3}H]UTP (20 nCi/nmol), and 0.5 μg of rifampicin in 45 μL of 0.04 M Tris-HCl buffer, 0.05 M KCl, and 5 mM $MgCl_2$. Samples were incubated 30 min at 37 °C, and RNA synthesis measured by the incorporation of labeled [^{3}H]UTP into acid-insoluble material (Lowery & Richardson, 1977a).

RNA Size Assay. The size of the RNA products made was analyzed by electrophoresis of the products of the RNA polymerase reaction mixture on polyacrylamide gels in 7 M urea (Maizels, 1973). The RNA polymerase reaction mixture was prepared as for the incorporation assays with the following modifications: ATP, GTP, and CTP were 20 nmol each; [^{3}H]UTP was 2 nmol and 100 nCi/nmol; rifampicin was not added. For preparation of a sample for electrophoresis, the RNA synthesis reaction was terminated by adding 10 μL of 1 M potassium acetate buffer (pH 5.0) and 10 μL of a solution containing 10 μg/mL DNAse, 150 μg/mL *E. coli* tRNA, and 0.1 M Tris-HCl buffer (pH 7.9). After 2 min at 37 °C, EDTA and sodium dodecyl sulfate were added to final concentrations of 15 mM and 0.2%, respectively. Protein was removed by extraction with an equal volume of phenol saturated with a solution containing 0.5 M Tris-HCl buffer (pH 8.0), 10 mM EDTA, 10 mM NaCl, and 0.5% sodium dodecyl sulfate. The phenol was reextracted with 100 μL of 0.1 M potassium acetate buffer (pH 5.0), and the pooled aqueous sample was extracted with 0.2 mL of $CHCl_3$–isoamyl alcohol (24:1). Two volumes of absolute ethanol were added to the aqueous layer, and the mixture was left to sit 15 h at −20 °C to precipitate the RNA. This step separates the RNA from most of the unincorporated nucleotides.

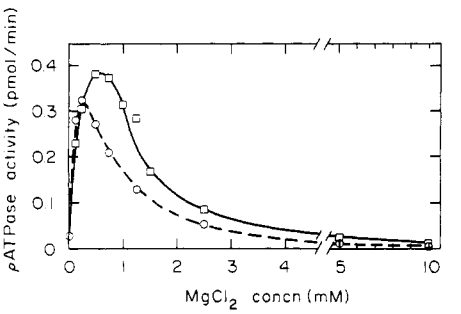

FIGURE 1: Dependence of ρATPase activity with T7 RNA on $MgCl_2$ concentration. The rate of hydrolysis of ATP catalyzed by ρ factor (ρATPase activity) with T7 RNA as cofactor was measured under the standard conditions given under Materials and Methods with the indicated amounts of $MgCl_2$ and with 1 mM ATP (□) or 0.4 mM ATP (○).

The labeled RNA precipitate was collected by centrifugation and dissolved in 20 μL of electrophoresis buffer (90 mM Tris, 90 mM boric acid, 4 mM EDTA, and 1% sodium dodecyl sulfate) containing 20% sucrose and 0.01% bromophenol blue. A 1-μL aliquot of each sample was assayed for acid-insoluble radioactivity so that equal amounts of radioactivity could be applied to each sample well. The gels (14 × 18 × 0.15 cm) were linear gradients of 7.5–3% acrylamide [ratio of acrylamide to bis(acrylamide) was 30:0.8] and 20–5% glycerol in electrophoresis buffer containing 7 M urea. After electrophoresis for 4 h at 100 V, the gels were impregnated with 2,5-diphenyloxazole (Bonner & Laskey, 1974) before being dried and exposed to X-ray film.

Results

Effect of $MgCl_2$ Concentration on Cofactor Activity of T7 RNA. From the studies made previously with poly(C) as a cofactor for the ATP hydrolysis activity of ρ, it was shown that the reaction is stimulated by $MgCl_2$ and that the stimulation is constant in the range 4–10 mM $MgCl_2$ (Lowery & Richardson, 1977a). When T7 RNA is the cofactor, the results are substantially different (Figure 1). Again, maximum activity depends on the presence of $MgCl_2$, but the optimum $MgCl_2$ concentration is lower than it is with poly(C), and the activity decreases significantly when the $MgCl_2$ concentration is increased above the optimum. With 1 mM ATP, the rate of hydrolysis is optimal in 0.6 mM $MgCl_2$ while in 10 mM $MgCl_2$ it is only 2% the rate in 0.6 mM $MgCl_2$. A similar effect of $MgCl_2$ concentration on ρATPase activity is found when isolated T4 RNA is used as a cofactor (data not shown). The fact that ρ can be fully active in 10 mM $MgCl_2$, when poly(C) is the cofactor, suggests that the divalent cation concentration is not exerting its effect by influencing the

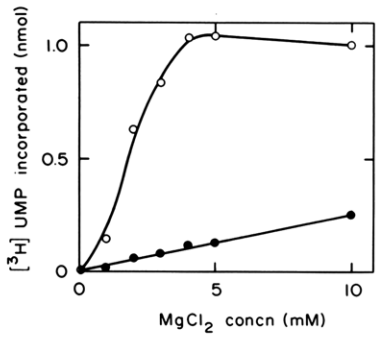

FIGURE 2: Yields of RNA transcribed for T7 DNA with *E. coli* RNA polymerase in the presence and absence of ρ factor as a function of $MgCl_2$ concentration. Reaction mixtures with the indicated amounts of $MgCl_2$ were assayed for RNA synthesis by using the nucleotide incorporation assay described under Materials and Methods. (○) RNA synthesized without ρ; (●) RNA synthesized with ρ.

structure or function of the ρ protein itself. On the other hand, it is known that the concentration of Mg^{2+} ions affects the stability of double-helical secondary structure in RNA (Cammack et al., 1970). Thus, the divalent cation concentration could be influencing the cofactor activity of T7 or T4 RNA by altering the structure of these RNAs. Since poly(C) has no double-helical secondary structure at pH 8.0, its cofactor activity is not inhibited by changes in conditions that increase the stability of secondary structures in RNA.

The results in Figure 1 show that the optimum $MgCl_2$ concentration for ρATPase activity with T7 RNA depends on the concentration of ATP. With 0.4 mM ATP, the optimum is 0.25 mM $MgCl_2$, while with 1 mM ATP, it is 0.6 mM $MgCl_2$. Since ATP binds Mg^{2+} ions, its presence will modulate the concentration of Mg^{2+} that will be free to bind to RNA. The optimum $MgCl_2$ concentration probably represents a balance between the requirement for the divalent cation and its inhibitory effect on the cofactor activity of the RNA. Since the optimum is directly proportional to the ATP concentration, the requirement may be for MgATP as the substrate for the hydrolysis reaction. It should be noted that because the concentration of ATP can indirectly affect the cofactor activity of the RNA by modulating the concentration of free Mg^{2+} ions, measurements of ρATPase activity with such RNAs as a function of ATP at a fixed total concentration of Mg^{2+} ions will not give reliable estimates of the K_m for ATP in the reaction.

$MgCl_2$ Affects Stringency of ρ Function in Termination. A simple test of the effect of $MgCl_2$ concentration on termination of T7 RNA synthesis by ρ action is to compare the yields of T7 RNA synthesized with *E. coli* RNA polymerase in the presence and absence of ρ under conditions where reinitiation is blocked (Figure 2). In the absence of ρ, the amount of RNA synthesized is constant from 4 to 10 mM $MgCl_2$. This suggests that RNA polymerase is as effective in transcribing T7 DNA in 4 mM $MgCl_2$ as it is in 10 mM $MgCl_2$. As expected, the yields of RNA synthesized in the presence of ρ are much lower than in the absence of ρ at all $MgCl_2$ concentrations tested. However, the yields with ρ present do not remain constant between 4 and 10 mM $MgCl_2$. Instead, there is nearly a 2.5-fold increase in the amount of RNA synthesized when the $MgCl_2$ concentration is increased over that range. Since the $MgCl_2$ concentration is not affecting the number of RNA chains initiated by RNA polymerase in that range, as evidenced by the constant yields in the absence of ρ, and since ρ is known to not influence greatly the number of RNA chains initiated (Roberts, 1969), the increase in yield of RNA synthesized in the presence of ρ should reflect an

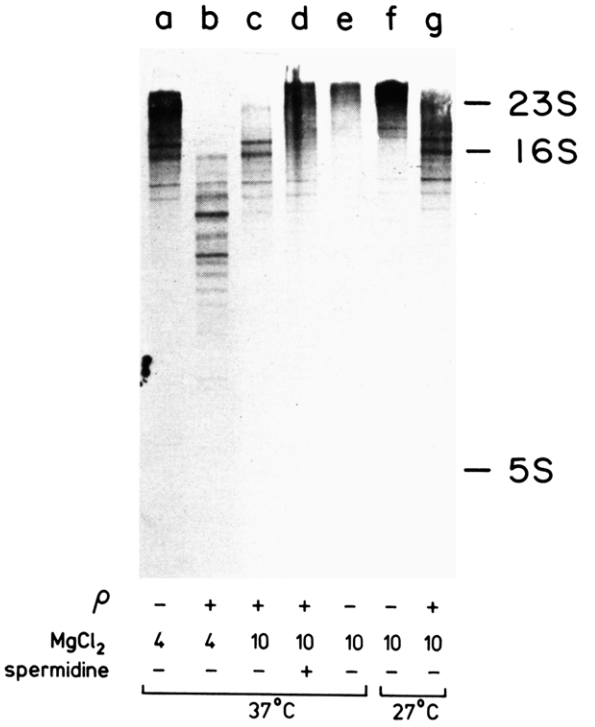

FIGURE 3: Radiofluorograph of T7 [^{3}H]RNA synthesized under different conditions after electrophoresis on polyacrylamide gel. The RNA samples analyzed were synthesized in the standard mixture for RNA size assay with the following conditions: (lane a) 4 mM $MgCl_2$, no ρ at 37 °C; (lane b) 4 mM $MgCl_2$, 0.6 μg of ρ at 37 °C; (lane c) 10 mM $MgCl_2$, 0.6 μg of ρ at 37 °C; (lane d) 10 mM $MgCl_2$, 2 mM spermidine, 0.6 μg of ρ at 37 °C; (lane e) 10 mM $MgCl_2$, no ρ at 37 °C; (lane f) 10 mM $MgCl_2$, no ρ at 27 °C; (lane g) 10 mM $MgCl_2$, 0.6 μg of ρ at 27 °C. The positions of *E. coli* RNA markers are indicated at the right.

increase in the average length of the RNA transcripts and thus a decrease in the stringency of ρ function in termination.

An analysis of the sizes of the RNA transcripts made in the presence of ρ in 4 and 10 mM $MgCl_2$ confirms this prediction (Figure 3). The ρ-terminated T7 RNA molecules synthesized in 4 mM $MgCl_2$ (lane b) migrate as several discrete bands corresponding to RNA chain lengths from 300 to 1500 nucleotides long, while those synthesized in 10 mM $MgCl_2$ (lane c) migrate in bands corresponding to RNA chain lengths from 600 to 3000 nucleotides long. The two most prominent bands in the T7 RNA synthesized in 4 mM $MgCl_2$ correspond to RNA molecules with lengths of 920 and 700 nucleotides. These could be transcripts initiated at the T7 A1 and A3 promoters at map positions 1.25 and 1.84 (Minkley & Pribnow, 1973; Stahl & Chamberlin, 1977; Studier et al., 1979) and terminated at a site between genes 0.3 and 0.7 (map position 3.64; a map unit is equivalent to 400 base pairs of T7 DNA; Rosenberg et al., 1979). Darlix (1974) has presented evidence for the existence of a ρ-sensitive transcription termination site between T7 genes 0.3 and 0.7. The band between the two prominent bands corresponds to an RNA with 800 nucleotides. It could be a transcript initiated at the A2 promoter (map position 1.55) and terminated at the site at map position 3.64. In contrast, the most prominent bands in the RNA synthesized in 10 mM $MgCl_2$ in the presence of ρ correspond to RNAs with 1500 and 1700 nucleotides. These could be transcripts initiated again at promoters A1 or A3 but terminated at map position 5.5, which is in the middle of gene 0.7. By this analysis, the specificity of ρ function in termination is clearly different in 10 mM $MgCl_2$ than it is in 4 mM $MgCl_2$.

Spermidine Inhibits ρ Function. Polyamines such as

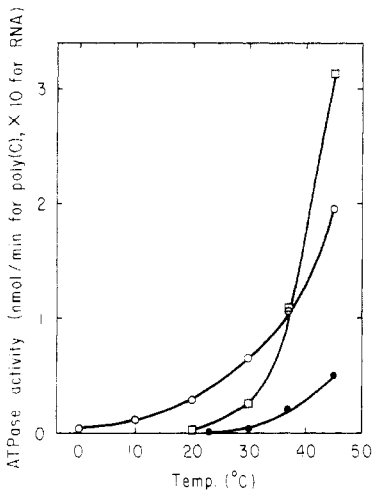

FIGURE 4: Effect of temperature on ρATPase activity. Rates of ATP hydrolysis with T7 and T4 RNA were measured by using a standard reaction solution containing 10 mM $MgCl_2$ incubated for 120 min. With poly(C) the mixtures contained 0.05 μg of ρ and were incubated 10 min. (●) T7 RNA; (□) T4 RNA; (○) poly(C).

spermidine are often added to transcription mixtures because they increase the yield of RNA synthesized. However, since polyamines are known to bind to RNA and can stabilize RNA secondary structure (Sakai & Cohen, 1976), they might inhibit ρ function like excess $MgCl_2$. When assays are performed with isolated T7 RNA in 10 mM $MgCl_2$ under the condition described in Figure 1, the rate of ATP hydrolysis in the presence of 2 mM spermidine is 20% the rate in the absence of spermidine (data not shown). In contrast, when poly(C) is the cofactor, the rate of ATP hydrolysis in 2 mM spermidine is 91% that in the absence of spermidine. Thus this polyamine is a potent inhibitor of ρATPase with T7 RNA as the cofactor. The results of one of the experiments presented in Figure 3 show that spermidine also inhibits ρ function in transcription termination. The two prominent bands in the RNA synthesized in 10 mM $MgCl_2$ with ρ (lane c) are not found in the RNA synthesized with 2 mM spermidine added (lane d). Instead the profile for that RNA is very similar to the profile of RNA synthesized in the absence of ρ (lane e).

Activation of RNA Cofactors by Temperature. Since the stability of secondary structure in an RNA is strongly dependent on temperature, the cofactor activity of T7 RNA might be enhanced significantly by increasing the temperature within a range that is compatible with retention of the structure of ρ protein. In order to distinguish between the effects of temperature on RNA cofactor activity and the ordinary thermal activation of the enzyme, we compared the results with T7 RNA with the effects of temperature on the activity with poly(C) (Figure 4). With poly(C), ρATPase activity increases ~2-fold for every 10 °C increase in temperature from 0 to 45 °C. This is a typical thermal activation (Q value) for an enzyme-catalyzed reaction (Dixon & Webb, 1958). With T7 RNA, activity in 10 mM $MgCl_2$ is nearly undetectable below 25 °C, and the extent of activation for an increase in temperature from 27 to 37 °C is 7.6-fold. A similar extent of activation is also found with T4 RNA as a cofactor (Figure 4). Thus we conclude that the greater enhancement of activity with temperature with these RNAs compared with poly(C) is a result of the effects of temperature on the secondary structure of the cofactors.

Because of the relationship that exists between ATP hydrolysis and termination activities of ρ, the strong effect of temperature on the hydrolysis could be reflected in an effect on the stringency of ρ function in termination; termination should be more stringent at a high temperature than at a low temperature. Results presented in Figure 3 show that this is true. Although many of the bands that are seen after electrophoresis of the RNA synthesized with ρ present at 37 °C (lane c) are also seen in the RNA synthesized at 27 °C (lane g), there is significantly more RNA in the size range between the positions of the 16 and 23S markers in the sample prepared at 27 °C than the one prepared at 37 °C. Hence temperature is an important variable in controlling ρ action.

Table I: Activation of RNA Cofactors for ρATPase in Solvents That Destabilize RNA Secondary Structure[a]

	rate of ATP hydrolysis (in pmol·min^{-1})				
	T4 RNA		T7 RNA		poly(C)
solvent addition	0.8 mM $MgCl_2$	10 mM $MgCl_2$	0.8 mM $MgCl_2$	10 mM $MgCl_2$	10 mM $MgCl_2$
none	535	20	383	6	643
10% methanol	925	35	646	22	570
10% ethylene glycol	593	7	571	8	661
10% glycerol	596	18	456	5	524
10% formamide	306	7	280	1	605
10% dimethyl sulfoxide	1320	66	1000	38	641
none, HCHO-treated, RNA			690	35	

[a] The rate of ATP hydrolysis was measured in standard reaction mixtures containing the indicated RNAs and with the indicated $MgCl_2$ concentrations. For measurement of the low-level rates with T4 and T7 RNA in 10 mM $MgCl_2$, the reaction mixtures were incubated 120 min. The reaction mixtures with poly(C) contained 0.05 μg of ρ and were incubated for 10 min. Formaldehyde-treated T7 RNA was prepared by the procedure of Lodish (1970). The reaction was initiated by adding 7.5 μL of 37% HCHO to 92.5 μL of solution containing 0.2 M NaCl, 9 mM Na_2HPO_4, and 1 mM NaH_2PO_4. After incubation at 37 °C for 11 min, the reaction was chilled on ice. Two volumes of ethanol were added, and after 20 min at −20 °C the RNA precipitate was collected by centrifugation. The precipitate was dissolved in 0.1 mL of 0.2 M NaCl and reprecipitated with ethanol. Finally, the precipitate was washed twice with ethanol and once with ether and dissolved in 0.5 mL of water. Residual ether was removed by a stream of nitrogen. The RNA was used immediately for the ATPase assays.

Activation of RNA Cofactors with Dimethyl Sulfoxide and by Treatment with Formaldehyde. If a decrease in the stability of the secondary structure in the RNA cofactor is the reason for activation of ρATPase by lowering the $MgCl_2$ concentration or increasing the temperature, it should be possible to increase the rate of ρ-catalyzed ATP hydrolysis with T7 RNA by adding solvents that destabilize RNA structure. A potent nucleic acid helix destabilizer is dimethyl sulfoxide (Helmkamp & Ts'o, 1961). The addition of this solvent at 10%, stimulates ρATPase up to 6-fold with T7 RNA and by substantial amounts with T4 RNA (Table I). However, the dimethyl sulfoxide has no effect on the reaction with poly(C), which is consistent with the interpretation that the solvent is exerting its effect on the structure of the RNA and not the protein (Table I). From tests with other organic solvents, it is found that methanol at 10% also causes some activation with T7 RNA and T4 RNA but not with poly(C), while formamide at 10% is inhibitory with all RNAs. Glycerol, which is commonly present in the reaction mixtures containing ρ, has no effect at 10%.

Another technique that has been used to reduce secondary structure in RNA is to treat it with formaldehyde (Lodish, 1970). Table I shows that T7 RNA that has been reacted with formaldehyde is significantly more active than untreated T7 RNA as a cofactor for ρATPase.

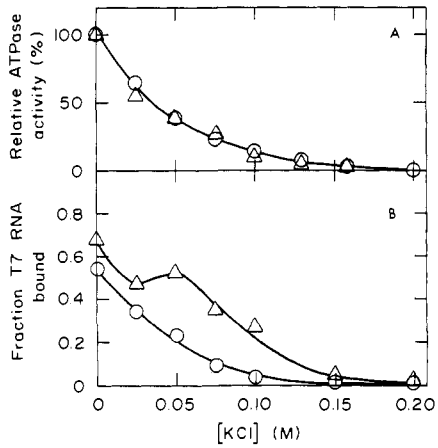

FIGURE 5: Effect of salt (A) on ρATPase activity with T7 RNA and (B) on binding of ρ to T7 [^{3}H]RNA. Rates of ρ-catalyzed ATP hydrolysis with T7 RNA were measured by using standard reaction solutions containing the indicated amount of KCl and either 10 mM $MgCl_2$ (○) or 0.6 mM $MgCl_2$ (△). The data are plotted as percent activity relative to the rates with no KCl present. These were 15 and 405 pmol/min for 10 and 0.6 mM $MgCl_2$, respectively. Binding of ρ to T7 RNA was measured by the amount of T7 [^{3}H]RNA retained on Millipore HA filters in the presence of ρ. The solutions containing in 0.1 mL of 0.04 M Tris-HCl buffer (pH 8.0) 0.1 μg/mL bovine serum albumin, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.2 μg of T7 [^{3}H]RNA (5000 cpm), 0.6 μg of ρ, and the indicated amounts of KCl and $MgCl_2$ were incubated 5 min at 37 °C, and 80-μL portions were filtered through a 13-mm Millipore HA filter and washed 2 times with 0.1-mL portions of a solution containing 0.04 M Tris-HCl (pH 8.0) and the same concentration of KCl and $MgCl_2$ used for the incubation. The amount of T7 [^{3}H]RNA retained was measured by counting the radioactivity on the dried filter. (○) 10 mM $MgCl_2$; (△) 0.6 mM $MgCl_2$.

Sensitivity of RNA Cofactor Activity to a Monovalent Salt. The function of ρ in transcription termination is known to be sensitive to the ionic strength of the reaction mixture (Richardson, 1970; Goldberg, 1970). With T7 DNA as the template, ρ functions most effectively between 0 and 0.025 M KCl, and its activity decreases as the KCl concentration is increased until there is virtually no detectable ρ-dependent termination in 0.2 M KCl. This effect of salt on termination has been correlated directly with its effect on the rate of ATP hydrolysis in the complete reaction mixture (Galluppi et al., 1976). Since the rate of ATP hydrolysis catalyzed by ρ with poly(C) as the cofactor is not sensitive to salt, it was suggested that the sensitivity of ρ function in the T7 transcription system could be reflecting an influence of ionic strength on the secondary structure of the RNA (Galluppi et al., 1976). It was assumed for this interpretation that ATP hydrolysis in the complete reaction mixture is reflecting the RNA-activated NTP hydrolysis activity of ρ. The results presented in Figure 5A indicate that this assumption is valid. When the rate of ATP hydrolysis catalyzed by ρ is measured as a function of KCl concentration with isolated T7 RNA as the cofactor, activity is maximum with no added KCl and decreases to nearly 0 as the KCl concentration is increased to 0.2 M. This effect does not depend strongly on the concentration of $MgCl_2$ used. Similar results have been found with T4 RNA as cofactor (data not presented). These results thus verify the previous conclusion that the salt effect on transcription termination is reflecting the ability of ρ to make a functional interaction with the nascent RNA molecule. However, the following experiments suggest that the major influence of ionic strength is on the affinity of ρ for the RNA and is not on the secondary structure of the RNA, as suggested previously.

The previous suggestion that the sensitivity of ρ function to ionic strength is reflecting an influence of ionic strength on the secondary structure of the RNA was based on evidence that R17 RNA structure becomes more compact as the concentration of monovalent cations increases (Gesteland & Boedtker, 1964). However, when divalent cations are also present, the effect of the monovalent cations is quite different. In solutions containing T7 RNA in 0.6 mM $MgCl_2$ at 37 °C, the absorbance of 260-nm light is 11% higher with 0.2 M KCl present than it is with 0.05 M KCl. Since hyperchromism in indicative of a *decrease* in double-helical secondary structure, this result suggests that in 0.6 mM $MgCl_2$ an increase in KCl concentration is destabilizing helical structures. A likely explanation for this reversal of effect is that as the KCl concentration increases the K^+ ions will displace Mg^{2+} ions that are more effective in stabilizing polynucleotide secondary structures. Hence, the effect of KCl on ρ function appears to be different from the effect of $MgCl_2$.

The evidence that the ionic strength affects the affinity of ρ for the RNA comes from measuring the amounts of T7 [^{3}H]RNA retained when solutions containing mixtures of ρ and T7 [^{3}H]RNA are passed through Millipore membrane filters. In a solution containing 0.6 mM $MgCl_2$ and 0.05 M KCl, 2 pmol of ρ in 0.1 mL can retain 50% of the RNA from a solution that originally contained 0.2 μg of T7 [^{3}H]RNA (Figure 5B). This result suggests that the apparent K_D of the T7 RNA–ρ complex is $\sim 2 \times 10^{-8}$ M. When these same amounts of ρ and T7 RNA are mixed and filtered in solutions containing increasing amounts of KCl, the fraction of RNA retained decreases to an undetectable level in 0.2 M KCl. Thus, in 0.2 M KCl, the K_D for the T7 RNA–ρ complex is $> 2 \times 10^{-6}$ M. These results suggest, therefore, that the effect of KCl on the binding affinity of ρ for T7 RNA could be the major factor responsible for the effect of salt on ρ functions. A complex between ρ and T7 [^{3}H]RNA can also be detected when the same amount of ρ and RNA are in 10 mM $MgCl_2$ and 0.05 M KCl, but, again, the fraction of RNA retained decreases to an undetectable level in 0.2 M KCl (Figure 5B). In this case the shape of the curve showing the retention of T7 RNA as a function of KCl concentration is similar to the shape of the curves showing the ρATPase activity in Figure 5A. It is not known why the shape of the curve showing the effect of KCl on binding in 0.6 mM $MgCl_2$ is different from the other curves in the range from 0 to 0.05 M KCl.

The RNA binding assay has also been used to determine whether the effects of $MgCl_2$ concentration, temperature, and dimethyl sulfoxide can be explained in terms of the ability ρ to bind to T7 RNA. The data in Figure 5B show that the $MgCl_2$ concentration does have an effect on the binding affinity, but the differences in retention of T7 RNA between 0.6 and 10 mM $MgCl_2$ are not as extreme as the differences in the rate of ATP hydrolysis in these two $MgCl_2$ concentrations, particularly at very low KCl concentrations. Bektesh (1979) has reported that ρ binds as well to λ *cro* mRNA at 4 °C as at 37 °C and that dimethyl sulfoxide at 10% has no effect on the binding. These observations have been repeated with T7 RNA (data not presented). Thus, the level of ATPase activity at low temperature with T7 RNA is not a result of an inability of ρ to bind to the RNA, and the stimulation of activity caused by the presence of dimethyl sulfoxide is not a result of an increase in the apparent affinity of ρ for T7 RNA. We conclude, therefore, that the step of the ATP hydrolysis reaction catalyzed by ρ that is most sensitive to the stability of the secondary structure is distinct from the primary interaction between ρ and RNA.

Discussion

The results presented in this paper indicate that the cofactor

activity of an RNA transcript is very sensitive to changes in reaction conditions that can affect the secondary structure of the RNA. The conditions that increase ρATPase activity with T7 RNA as a cofactor are those that destabilize double-helical structures within the RNA. Our results confirm and extend the conclusion made previously by Adhya et al. (1979), based on the effects of nucleotide analogues on ρ functions—both termination and ATP hydrolysis—during transcription of T3 DNA. They found that replacement of GTP with ITP, which will give RNA molecules with weaker secondary structures, increased significantly the stringency of termination and the rate of ATP hydrolysis, while the replacement of CTP with BrCTP, which would give RNA molecules with a more stable secondary structure, gave the opposite results. Since the studies of Adhya et al. (1979) were done in complete reaction mixtures, their data could be interpreted in terms of the stabilities of either RNA–RNA helices or RNA–DNA helices. However, the results presented in this paper correlate effects of helical stabilities on ρATPase activity with isolated RNA. For this reason, we believe that the relative stability of RNA–RNA helices is important for ρ function in termination.

Our results also show that the specificity of ρ action in terminating transcription can be changed significantly by altering the reaction conditions. Thus, special attention should be given to the $MgCl_2$ concentration and temperature used for transcription studies involving ρ. It should also be noted that the dependence of ρ function on temperature could have an important physiological effect on the regulation of gene expression in bacteria. In fact, it has been found by D. Friedman and his co-workers (personal communication) that the expression of λ genes by read through of ρ-dependent termination sites is measurably higher at low temperature than at high.

The results of previous studies with synthetic homo- and copolymers showed that single-stranded pyrimidine-rich RNA polymers are the most effective ρATPase cofactors and that totally double-helical molecules are inactive (Lowery & Richardson, 1977b). T7 RNA undoubtedly has a complex structure that includes both double-helical and single-stranded regions. It is not known yet whether a single-stranded region is needed for the initial interaction between ρ and the RNA or whether the single strandedness or the ability to become single-stranded is important for some other step of the ρ–RNA interaction. It has been proposed that the ATP hydrolysis reaction is coupled to a winding of RNA around the outside of ρ (Galluppi & Richardson, 1980). Thus, the ability to dissociate secondary structure interactions in the RNA could be essential to allow the winding reaction to occur. Since binary complexes between T7 RNA and ρ form almost as readily in 10 mM $MgCl_2$ as in 0.6 mM $MgCl_2$ (see Figure 5B) and since ρ binds as well to RNA at 4 °C as at 37 °C (Bektesh, 1979), it appears that these reaction conditions are exerting their effects on ρATPase activity at a step after the initial binding reaction, perhaps on the ATP-dependent winding of RNA around ρ. It will be of interest to demonstrate whether ρ can actually mediate the dissociation of secondary structures in RNA.

The evidence presented in this paper indicates that the effect of ionic strength on ρ function is exerted through the ability of ρ to bind to the RNA. T7 RNA does not bind to ρ in 0.2 M KCl and has no cofactor activity under these conditions. In contrast, the binding of ρ to poly(C) is insensitive to the ionic strength (Galluppi & Richardson, 1980) as is the cofactor activity of this polymer (Galluppi et al., 1976). Since the binding of ρ to poly(U), another single-stranded polymer of pyrimidine nucleotides, is sensitive to salt concentration, it was concluded (Galluppi & Richardson, 1980) that ρ must be able to make strong, nonionic interactions with cytidylate residues to give poly(C) its very high binding affinity, while the binding of ρ to RNA molecules that are not especially rich in cytidylate residues must involve ionic interaction to stabilize the complex. We conclude therefore that the binding of ρ to T7 RNA is in the latter category and it is this dependence on ionic interactions that accounts for the effect of salt concentration on ρ activity in transcription termination.

Acknowledgments

We thank Dr. Thomas Blumenthal for a critical reading of the manuscript.

References

Adhya, S., & Gottesman, M. (1978) *Annu. Rev. Biochem. 47*, 967–996.

Adhya, S., Sarkar, P., Valenzuela, D., & Maitra, U. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1613–1617.

Bektesh, S. L. (1979) Ph.D. Dissertation, Indiana University, Bloomington, IN.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634–4638.

Cammack, K. A., Miller, D. S., & Grinstead, K. M. (1970) *Biochem. J. 117*, 745–755.

Chamberlin, M., & Berg, P. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 81–94.

Darlix, J. L. (1974) *Biochimie 56*, 693–701.

Dixon, M., & Webb, E. C. (1958) *Enzymes*, p 162, Academic Press, New York.

Finger, L. R., & Richardson, J. P. (1981) *Biochemistry* (in press).

Galluppi, G., & Richardson, J. P. (1980) *J. Mol. Biol. 138*, 513–539.

Galluppi, G., Lowery, C., & Richardson, J. P. (1976) in *RNA Polymerase* (Losick, R., & Chemberlin, M., Eds.) pp 657–665, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Gesteland, R. F., & Boedtker, H. (1964) *J. Mol. Biol. 8*, 496–501.

Goldberg, A. R. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 157–161.

Helmkamp, G. K., & Ts'o, P. O. P. (1961) *J. Am. Chem. Soc. 83*, 138–142.

Howard, B., & deCrombrugghe, B. (1976) *J. Biol. Chem. 251*, 2520–2524.

Lodish, H. F. (1970) *J. Mol. Biol. 50*, 689–702.

Lowery, C. L., & Richardson, J. P. (1977a) *J. Biol. Chem. 252*, 1375–1380.

Lowery, C. L., & Richardson, J. P. (1977b) *J. Biol. Chem. 252*, 1381–1382.

Lowery-Goldhammer, C., & Richardson, J. P. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2003–2007.

Maizels, N. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3585–3589.

Minkley, E. G., & Pribnow, D. (1973) *J. Mol. Biol. 77*, 255–277.

Richardson, J. P. (1970) *Cold Spring Harbor Symp. Quant. Biol. 35*, 127–133.

Richardson, J. P., & Conaway, R. (1980) *Biochemistry 19*, 4293–4299.

Roberts, J. W. (1969) *Nature (London) 224*, 1168–1174.

Roberts, J. W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 247–271, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Rosenberg, A. H., Simon, M. N., Studier, F. W., & Roberts, R. J. (1979) *J. Mol. Biol. 135*, 907–915.
Sakai, T. T., & Cohen, S. S. (1976) *Prog. Nucleic Acid Res. Mol. Biol. 17*, 15–42.
Stahl, S. J., & Chamberlin, M. J. (1977) *J. Mol. Biol. 112*, 577–601.
Studier, F. W., Rosenberg, A. H., Simon, M. N., & Dunn, J. J. (1979) *J. Mol. Biol. 135*, 917–937.
Thomas, C. A., Jr., & Abelson, J. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., & Davies, D. R., Eds.) pp 553–561, Harper and Row, New York.

Catalytic Mechanism of Phenylalanyl-tRNA Synthetase of *Escherichia coli* K10. Different Properties of Native and Photochemically Cross-Linked $tRNA^{Phe}$ Can Be Explained in the Light of tRNA Conformer Equilibria†

Eggehard Holler,* Mireille Baltzinger, and Alain Favre‡

ABSTRACT: Kinetic and equilibrium constants of phenylalanylation, AMP-dependent deacylation, and AMP-independent deacylation by phenylalanyl-tRNA synthetase of *Escherichia coli* K10 have been measured for $tRNA^{Phe}$ that was cross-linked by UV irradiation between uridine in position 8 and cytidine in position 13. The association of the aminoacylated tRNA with enzyme was investigated by stopped-flow techniques. The results are compared with those for native tRNA and Phe-tRNA. (1) Formation of the enzyme·substrate complexes follows dissociation constants and Michaelis–Menten complexes with 25-fold higher values for cross-linked in comparison to native tRNA. (2) Catalytic rate constants are the same for phenylalanylation of native and cross-linked $tRNA^{Phe}$. (3) Michaelis–Menten constants for AMP-dependent and -independent deacylation of Phe-tRNA have similar values for native and cross-linked $tRNA^{Phe}$. (4) The catalytic rate constant of AMP-dependent deacylation is 10^3-fold reduced in comparison to that of native Phe-tRNA, while that of AMP-independent deacylation is twice the value for native Phe-tRNA. (5) The kinetics of enzyme·Phe-tRNA complex formation are described by the same minimal reaction scheme as for native Phe-tRNA, a rapid binding equilibrium at the tRNA-specific binding site of the enzyme and a rate-determining binding at the phenylalanine-specific binding site of the enzyme [Holler, E. (1980) *Biochemistry 19*, 1397–1402]. The complex at the tRNA-specific site has a dissociation constant of 0.35 μM. This is similar to the K_m values for deacylation. Rate constants for dissociation of native and cross-linked Phe-tRNA from the phenylalanine-specific binding site have identical values. The same values have been found for the catalytic rate constants of phenylalanylation. The association of native Phe-tRNA to the Phe-specific binding site follows a rate constant that has a 25-fold higher value than that for cross-linked Phe-tRNA. Whereas for native Phe-tRNA the rate constants are the same for the association at the Phe-specific site and for the AMP-dependent deacylation, that of the association of cross-linked Phe-tRNA is 46-fold higher than the value of the rate constant for AMP-dependent deacylation. All the differences between native and cross-linked $tRNA^{Phe}$ are accounted for by a unifying model which assumes cross-link and phenylalanylation dependent conformer equilibria and which assumes the rate-limiting steps of phenylalanylation and AMP-dependent deacylation to be a conformational change during dissociation–association of Phe-tRNA at the phenylalanine-specific binding site of the enzyme.

The present work was initiated for two reasons. (1) Near-UV-induced growth delay of *Escherichia coli* has been discovered to be coupled to an intramolecular 8–13 cross-linking of tRNAs (Thomas & Favre, 1975; Ramabhadran & Jagger, 1976). Mutants have been grown which no longer exhibit the growth delay and which are deficient in 4-thiouridine, the chromophore responsible for cross-linking (Thomas & Favre, 1977). $tRNA^{Phe}$ has been found to play a key role. Upon irradiation of crude in vitro systems, initial rates of phenylalanylation were decreased to 5% of that of aminoacylation of other tRNAs by using conventional assay conditions (Carrê et al., 1974). We present here kinetic parameters of phenylalanylation and AMP-dependent deacylation for cross-linked $tRNA^{Phe}$ in comparison to that of native $tRNA^{Phe}$. The results are self-explanatory and support previous conclusions (Thomas & Favre, 1977).

(2) The reaction of cross-linking has been found to be inhibited by formation of a complex between $tRNA^{Phe}$ and phenylalanyl-tRNA synthetase of *E. coli* (Favre et al., 1979). Complex formation has been shown to favor a rearrangement of $tRNA^{Phe}$ that decreases the probability of occurrence of a transition state necessary for 8–13 link formation. Reciprocally, the number of conformations of $tRNA^{Phe}$ is expected to be decreased after cross-linking. The present work examines the effect of cross-linking on various steps of the catalytic reactions of phenylalanyl-tRNA synthetase in comparison to that of native $tRNA^{Phe}$. The results are interpreted by a unifying concept, which allows for conformer equilibria of

† From the Institut für Biophysik und Physikalische Biochemie, der Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany. *Received July 10, 1980.* This work was supported by a NATO grant to E.H. and A.F. M.B. received an EMBO postdoctoral fellowship.
‡ Present address: Institut de Recherches en Biologie Moléculaire, Paris, 75005 France.

0006-2960/81/0420-1139$01.25/0