Characterization of the Nucleoside Triphosphate Phosphohydrolase (ATPase) Activity of RNA Synthesis Termination Factor ρ

II. INFLUENCE OF SYNTHETIC RNA HOMOPOLYMERS AND RANDOM COPOLYMERS ON THE REACTION*

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The ability of various kinds of RNA molecules to activate the ATP hydrolysis reaction catalyzed by the ρ transcription termination factor from Escherichia coli has been studied. The most active RNA polymers are those containing cytidylic residues and very littleordered structure. Free poly(C) is the most active homopolymer; it is 45 times more active than poly(U), which is the only non-cytidine containing RNA that has detectable activity. Poly(C) has no activity when complexed with poly(I) or when the chain lengths are shorter than 22 nucleotides long. Although cytidylic residues are important they need not be frequent; a random copolymer of uridine and cytidine nucleotides with as few as 1 cytidylic residue out of 20 is as active as poly(C). The extent of activation with poly(C) depends on the ratio of ρ to poly(C). Poly(C) becomes saturated with ρ at a ratio of 1.8 pmol poly(C)/mg of ρ, which is equivalent to one ρ monomer/27 nucleotides. A further increase in this ratio leads to a reduction in ρ activity. Decreasing the length of the poly(C) does not alter the observed saturation value but does decrease the rate of ATP hydrolysis when the RNA is in excess. The possible relevance of these results to ρ termination activity is discussed.

The ρ transcription termination factor of Escherichia coli has an RNA-dependent nucleoside triphosphate phosphohydrolase (ATPase) activity (1) which is required for its termination activity (2, 3). In the presence of poly(C) it can catalyze the hydrolysis of ATP to ADP and Pi at a rate exceeding 150 μmol/min/mg (4). Initial studies indicated that the extent of activation depended on the RNA used and that poly(C) was a particularly effective activator (1). In this paper we report studies describing the nature of the RNA requirement for the ρ ATPase activity. These studies provide some potential clues to the mechanism of ρ action in transcription termination.

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MATERIALS AND METHODS

Procedures used for the isolation and assay of ρ factor have been described in the preceding paper (4). The synthetic RNA and DNA polymers were purchased from Miles Laboratories, Inc. except where indicated otherwise.

Partially purified primer-independent polynucleotide phosphorylase from Micrococcus luteus was purchased from P-L Biochemicals and purified further by gel filtration chromatography on Sephadex G-200. This enzyme was used to synthesize copolymers of poly(A,C) and poly(U,C) containing different proportions of the two nucleotides. These polymers were synthesized in reaction mixtures containing 0.1 M Tris/HCl buffer (pH 9.0), 5 mM MgCl₂, 0.4 mM EDTA, 80 μg of bovine serum albumin/ml, 8 mM nucleoside diphosphate (Sigma Chemical Co.), and 3.5 milliunits of polynucleotide phosphorylase in 25 μl. After incubation for 100 min at 37°, 0.225 ml of H₂O was added and the amount of polymer synthesized was determined by assay of the amount of P₁ released in a 0.10-ml portion using a colorimetric assay for P₁, described previously (4). The rest of the sample was heated to 70° for 5 min and stored frozen until used. About 25 nmol of nucleotides were incorporated in each reaction mixture for nearly all the ADP plus CDP and UDP plus CDP mixtures.

Low molecular weight poly(C) was prepared by alkaline degradation of a commercial poly(C) preparation. Two milligrams of poly(C) in 1 ml of 0.1 N NaOH was incubated for 2 h at 37°. The hydrolysis of intranucleotide diester bonds was terminated by reducing the pH to 3 with 1 N HCl. This solution was incubated further for 1 h at 37° to hydrolyze the cyclic 2':3'-phosphodiester bonds. After neutralization in 10 N NaOH and adding Tris/HCl buffer to bring the pH to 7.5, the solution was treated with 15 μg of Escherichia coli alkaline phosphatase (Worthington, grade BAPP) for 2 h at 37°. The phosphatase-treated, low molecular weight poly(C) molecules were purified by phenol extraction, using, successively, ether to remove residual phenol and bubbling N₂ to remove the ether. Fractionation of this mixture on the basis of size was accomplished by chromatography on a Sephadex G-75 column (1 x 50 cm) in a solution containing 0.01 M Tris/HCl buffer (pH 7.9), 0.1 M NaCl, and 10 μM EDTA at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected.

The number average chain lengths of the oligo(C) in selected fractions was determined from the ratio of cytidine to cytidylic acid present after complete alkaline digestion. A portion containing about 0.8 A₂₆₀ unit was heated 20 min at 100° in 0.5 N NaOH, neutralized with HCl and Tris/HCl, and applied to a quaternary aminoethyl (QAE)-Sephadex-acetate column (0.4 x 13 cm). The cytidine and cytidylic acid isomers were eluted successively with 0.01 M Tris/HCl buffer (pH 7.9), 0.1 M NaCl, and 10 μM EDTA at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected.

The number average chain lengths of the oligo(C) in selected fractions was determined from the ratio of cytidine to cytidylic acid plus cytidine present after complete alkaline digestion. A portion containing about 0.8 A₂₆₀ unit was heated 20 min at 100° in 0.5 N NaOH, neutralized with HCl and Tris/HCl, and applied to a quaternary aminoethyl (QAE)-Sephadex-acetate column (0.4 x 13 cm). The cytidine and cytidylic acid isomers were eluted successively with 0.01 M HCl, and the amounts were determined from the absorbance at 280 nm.

RESULTS

Activity with Various RNAs—A number of different poly-
nucleotides have been tested for their ability to activate the pATPase. The best activators are ribopolymers that are rich in cytidine (Table I). However, the results with polyc(G, A, U, etc.) indicate that the presence of cytidylate residues in RNA is not a sufficient condition for activity. Also, the presence of cytidylate residues in polynucleotides tested, including poly(dC), are active.

**Dependence of pATPase Activity on Amount of Poly(C)** - The activation of the pATPase depends on the ratio of poly(C) to p. For maximum activity there must be at least 1.5 pmol of poly(C)/ng of p (Fig. 1). One-half maximal activity occurs with 0.55 pmol of poly(C)/ng of p. The reason for the sigmoid shape of the poly(C) activation curve becomes evident when the activity as a function of p is assayed with different fixed amounts of poly(C) (Fig. 2). With 5 pmol of poly(C), the activity increase is proportional to the amount of p up to ~25 ng of p and then decreases. Thus when the ratio of p to poly(C) increases above the critical value of about 1.8 ng of p/pmol of poly(C) the excess p inhibits the reaction. This critical ratio is the reciprocal of the ratio of poly(C) to p that gives half-maximal activity on the poly(C) activation curve (Fig. 1). In molecular terms, this ratio is equal to one p monomer (M, = 50,000; Ref. 5) for every 27 nucleotides.

**Dependence of pATPase Activity on Length of Poly(C)** - Hydrolysis of polyc with alkali or by action of pancreatic ribonuclease destroys its ability to activate pATPase (1). The minimum size of poly(C) needed for activity was determined by assaying fractions of partially hydrolyzed poly(C) that had been chromatographed on Sephadex G-75. The assays were performed using a constant amount of the different size polymers at a level just sufficient to give full activity with unhydrolyzed poly(C). The results (Fig. 3) indicate that the activity falls rapidly as the average size decreases below a chain length of 45 nucleotides until there is no activity with the fractions containing poly(C) with an average length of 22 nucleotides or less.

The poly(C) fractions with average chain lengths of 40, 33, and 20 nucleotides have been analyzed further for their ability to activate pATPase as a function of amount (Fig. 4). The poly(C) gives a saturation curve that is very similar to the curve for untreated poly(C). In assay mixtures containing 20 ng of p factor, 11 pmol of poly(C) is sufficient to give half-maximal activity. The saturation curve for poly(C) is less distinct, which may be because it contains a mixture of polymers with different intrinsic activities. However, there appears to be a saturation with the major component and the amount of this major component needed to reach half-maximal saturation with 20 ng of p is 10 pmol. The ratio of p to poly(C) is very similar to the "critical ratio" of p to poly(C) found with the undegraded polymers that are on the average 10 times larger than poly(C). Thus this "critical ratio" is independent of the size of poly(C).

However, the size does affect the maximum rate of ATP hydrolysis when p is saturated with the polymers. This maximum rate is approximately 2-fold greater for poly(C) than for

| Polymer            | nmol | 32P released |
|--------------------|------|--------------|
| Poly(C)            | 9.1  | 9.1          |
| Poly(U)            | 0.20 | 0.20         |
| Poly(A)            | <0.06| <0.06        |
| Poly(G)            | <0.06| <0.06        |
| Poly(I)            | <0.01| <0.01        |
| Poly(C, U)         | 8.5  | 8.5          |
| Poly(A, G)         | 7.8  | 7.8          |
| Poly(C, A, U, G)   | <0.01| <0.01        |
| Poly(C, G, A, U)   | 8.2  | 8.2          |
| Poly(dC)           | <0.04| <0.04        |
| Poly(dT)           | <0.06| <0.06        |
| Poly(dA)           | <0.06| <0.06        |
| Poly(dG)           | <0.06| <0.06        |

![Fig. 1 (left). Dependence of pATPase activity on the amount of poly(C). ATPase activity was determined by measuring the release of 32P, from [γ-32P]ATP (2,000 cpm/mmol) was determined using standard reaction mixtures containing, in 0.1 ml, 6.6 nmol of polynucleotide and 20 ng of p. Incubation was for 15 min at 37°.](http://www.jbc.org/)

![Fig. 2 (right). Dependence of pATPase activity on amount of p with different amounts of poly(C).](http://www.jbc.org/)

![Fig. 3. Dependence of pATPase activity on the size of poly(C). ATPase activity was determined by measuring the release of 32P from [γ-32P]ATP in 0.1 ml of standard assay mixture using the indicated amounts of p and poly(C) (1 milliunit of ATPase is 1 nmol of ATP hydrolysed/min).](http://www.jbc.org/)
the major component of poly(C)$_{25}$, which suggests that the size of the polymer in this range of 20 to 45 nucleotides long can affect a rate-limiting step of ATP hydrolysis catalyzed by $\rho$. Further studies (not shown here) using poly(C) that has been degraded to a lesser extent and fractionated on a Sephadex G-200 column indicate that after the sharp increase in activity in the size range of 20 to 45 nucleotides there is a gradual 2-fold further increase in the maximum rate of ATP hydrolysis when the poly(C) molecules increase in average size in the range from 45 to over 1000 nucleotides in length.

It is possible to detect activity with high amounts of poly(C)$_{25}$. With 20 ng of $\rho$, 0.03 milliunits (nanomoles of ATP hydrolyzed/min) is found with 100 pmol of poly(C)$_{25}$ (Fig. 4). When the poly(C)$_{25}$ is increased to 300 pmol, 0.08 milliunits of ATPase activity is detected. It is likely that this low level of activity is from the presence of trace amounts of the active, longer polymers in the poly(C)$_{25}$ fraction.

**Activity of Copolymers Containing Various Proportions of Cytidylate Residues**—When present in large excess, poly(U,C) and poly(A,C) are as effective as poly(C) in activating $\rho$-ATPase (Table I). Since poly(A) has no activity and poly(U) has very little activity, it is of interest to determine what fraction of the residues need to be cytidylate to give a polymer that is as effective as poly(C). Copolymers with different proportions of nucleotides in random sequences can be synthesized by action of polynucleotide phosphorylase using different proportions of nucleoside diphosphates (6). The activities of different A,C and U,C copolymers at an amount just sufficient to saturate the $\rho$ activity with poly(C) are plotted in Fig. 5 as a function of the fraction of CDP present in the polynucleotide synthesis mixture. (The activities of different A,C and U,C copolymers are shown in Fig. 5 as a function of the fraction of CDP present in the polynucleotide synthesis mixture.)

**Inactivation of Poly(C) by Interaction with Poly(Z) by Poly(I)**—Poly(C) is able to form a stable double helical complex with poly(I) when the two polymers are allowed to anneal under the appropriate conditions (7). By mixing increasing amounts of poly(I) with poly(C), under these conditions, all the ATPase activity is eliminated even before there is enough poly(I) to complex all the poly(C) (Fig. 7). If, however, the poly(I) is mixed with the poly(C) but not allowed to anneal with it, full activity remains. Thus the poly(I) does not appear to inhibit $\rho$ directly. This experiment suggests that it inhibits the reaction by complexing with the poly(C). The fact that the shape of the inactivation curve is sigmoid is probably due to the decrease in amount of free poly(C) caused by the addition of poly(I). This reduces the poly(C) to $\rho$ ratio below the critical value, thus causing a rapid drop in activity.

**DISCUSSION**

The activation of $\rho$-ATPase depends on the amount, composition, size, and structure of the RNA. The most active polymers all contain cytidylate residues. Poly(U) also has some activity, but it is less than 2% as effective as poly(C). However, the fact that a U,C copolymer with as few as 1 cytidylate residue out of 20 is as active as poly(C) suggests that the proportion of cytidylate residues need not be very high for good activity. Since the polymers of uridine and cytidine are more effective than polymers of adenosine and cytidine, it is possible that a minimum stretch of pyrimidine residues containing cytidine nucleotides is required for activity. Alternatively, the activity may be affected by the differences in secondary structure between the purine-containing A,C copolymers and the all pyrimidine U,C copolymers.

One aspect of secondary structure does appear to be very important; when poly(C) is complexed with poly(I) it has no
The finding that the ATPase activity of ρ factor can be limited by the amount of poly(C) present is not surprising. The ρ monomer has a molecular weight of 50,000 A globular protein of that size would have an effective diameter of about 50 Å. Thus poly(C) could become physically saturated with one ρ monomer per 15 to 20 nucleotides. The "critical ratio" of ρ to poly(C) for the ATPase reaction is about one ρ/27 nucleotides. However, this could be high by as much as a factor of 2 because of uncertainties in determining the amount of ρ used. This is measured spectrophotometrically assuming Α_{260} = 1/mg/ml; the actual value is not known. Thus the "critical ratio" is close to that expected for close packing of ρ monomers on a single-stranded polynucleotide. The surprising finding is that when the ratio of ρ to poly(C) is increased above the "critical ratio" there is a decrease of ATPase activity. One explanation for this effect is that ρ molecules that are unable to bind to RNA might bind instead to other ρ molecules, thus altering their activity. ρ factor is known to oligomerize and probably exists primarily as tetramer in the last step of purification (5, 8). It is not known yet whether ATP hydrolysis is an activity of the monomeric protein or of an oligomeric form. Minkley presented evidence that an oligomeric form is active in termination based on the observation that the dependence of termination of ρ concentration shows cooperative activation that parallels the ability of ρ to sediment as an oligomer (8). He found that ρ at a concentration less than 0.1 μg/ml did not cause termination of transcription of λDNA. With excess poly(C), ρATPase activity is directly proportional to the amount of ρ to less than 5 ng/ml (Fig. 2). Thus, ρATPase does not seem to be subject to cooperative activation in the concentration ranges that apparently affects ρ termination. Since Minkley found that ρ sediments as a monomer when its concentration is less than 0.3 μg/ml, it appears that ρ can function as a monomer in the ATPase reaction.

Although the "critical ratio" may be important in understanding the mechanism of activation of ρATPase by poly(C), it is not clear whether it has any relevance to the mechanism of ρ-dependent termination of RNA synthesis, since it is not yet known whether termination is inhibited by excess ρ factor.

The size of the poly(C) in the range of 22 to 45 nucleotides appears to affect the rate-limiting step in ρ hydrolysis, not the amount of ρ needed to saturate the poly(C). This effect of size on the rate could be a reflection of the mechanism of ρ action on the poly(C). For instance if the hydrolysis of ATP is coupled to a movement of ρ along the poly(C) a rate-limiting step in the overall reaction could be the dissociation of the ρ from the poly(C) once it reaches the end of the polymer. Thus the shorter the polymer the less each ρ molecule will be able to hydrolyze before it reaches an end and has to wait to dissociate and subsequently reassociate in a region that allows ATP hydrolysis. Consequently, the shorter the poly(C) the less ATP will be hydrolyzed by ρ per unit of time. Although other explanations could be suggested, this one is of interest because of the possibility that a directed movement of ρ factor along a nascent RNA from a ρ recognition site on the RNA to the RNA polymerase could be a crucial process in ρ-mediated termination of transcription.

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