Substrate, Metal and Template Effects on Inhibition of Bacteriophage-Q β Ribonucleic Acid Polymerase by Ortho- and Pyro-phosphate

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Two reactions of bacteriophage-Q β RNA polymerase with synthetic templates were characterized and used to study the effects of substrate, metal and template on inhibition by P_i and PP_i. Analysis of the poly(C)-dependent reaction yielded results on kinetics, GTP-dependence, preference for Mn²⁺ over Mg²⁺, and Michaelis constants for template similar to those in the literature. New data are provided for the poly(U₂,C)-dependent reaction. Our results suggest that GTP and Mn²⁺ can form relatively stable complexes with the polymerase and that such complexes change the interaction of the enzyme with the inhibitors, P_i and PP_i.

The study of RNA synthesis *in vitro* is made complex by the numerous components of polymerase reactions, including four substrates, a template and/or primer nucleic acid, a bivalent-metal cofactor, and additional regulatory proteins (Mueller, 1971; Franze de Fernandez *et al.*, 1972; Stevens, 1972; Hoffman & Niyogi, 1973). Further, RNA polymerases, either DNA- or RNA-dependent, are multi-subunit structures (Burgess, 1969; Kamen, 1970; Landers *et al.*, 1974) that may contain more than one substrate-binding site (Wu & Goldthwait, 1969; Hori, 1974). The functions of these components in normal RNA synthesis have not been fully delineated. We have been interested in the inhibition of RNA synthesis, thus adding another factor, the inhibitor, to the reaction.

Numerous inhibitors of bacteriophage-Q β RNA polymerase have been reported. These include aurintricarboxylic acid (Blumenthal & Landers, 1973), rugulosin (Nakamura *et al.*, 1971) and the iodoacetylenes (Haruna *et al.*, 1970). Haruna *et al.* (1970) used this enzyme system to search for a specific inhibitor of RNA replicase that could serve as an antiviral drug. Yet the effect of substrate, bivalent cations and template on inhibition of the phage-Q β RNA polymerase by these or other compounds has not been elucidated. To shed light on this we have chosen simple reactions of the enzyme, using poly(C) or poly(U₂,C) as template and requiring only one or two nucleoside triphosphates for substrate. Further, these reactions will occur without added protein factors and can use either Mn²⁺ or Mg²⁺ as cofactor. The inhibitors examined were P_i and PP_i, the latter being a product of the nucleotidyltransferase reaction. We report here the characterization of the poly(C)- and poly(U₂,C)-dependent reactions and the effects of metal ion, GTP and template on inhibition.

A preliminary account of part of this work has been presented (Brooks *et al.*, 1975).

Materials and Methods

Bacteria and virus

Colonies of *Escherichia coli* Q13 (a gift from Dr. S. Spiegelman, Columbia University, U.S.A.) and *E. coli* K12 (λ –, Su-1) (A.T.T.C. 23736) able to grow in the presence of 0.2M-MgSO₄ were isolated as described by Gschwender & Hofschneider (1969). Bacteriophage Q β (am 12), a replicase overproducer (Horiuchi & Matsushashi, 1970), was a gift from Dr. K. Horiuchi (The Rockefeller University, U.S.A.). Stocks of phage were obtained by infecting 500ml cultures of *E. coli* K12 in Kamen's (1972) medium at net A_{660} of 0.08–0.16 with single plaques of virus, growing for 4h at 37°C and lysing with 25ml of 0.1M-EDTA/1M-Tris/HCl, pH 7.5 at 25°C. Phage was titred and used to infect additional 500ml cultures to produce enough phage to infect 10 litres of *E. coli* K12 (10⁹ cells/ml) at a multiplicity of 1 plaque-forming unit/cell in a Microferm fermenter (New Brunswick Scientific, New Brunswick, NJ, U.S.A.). After 3h of growth at 37°C, during which the cultures were stirred at 400rev./min with a single blade and aerated with 16 surface litres of air/min in the presence of 0.5ml of Antifoam A (Dow Corning, Midland, MI, U.S.A.), 10ml of chloroform and 585ml of lysing solution (as above) were added. Virus stock was stored at 4°C.

E. coli Q13 was grown to 5 × 10⁸ cells/ml in 15 litres of Kamen's (1972) medium in the Microferm fermenter with 1.5ml of Antifoam A. Phage was added (multiplicity of infection = 20 plaque-forming units/cell) without agitation and then incubation was continued for 2h as above. Infected cells were col-

lected by centrifugation (12100g for 5 min at 4°C) and stored at -20°C.

Enzyme preparation

Bacteriophage-Q β RNA polymerase was purified from infected *E. coli* Q13 as described by Eoyang & August (1971) with the omission of hydroxyapatite chromatography. The final product showed four bands on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and had a specific activity of over 3000 units/mg of protein with Mn²⁺ in the poly(C)-dependent assay; 1 unit is the incorporation of 1 nmol of GMP in 20 min. Enzyme was stored at 4°C in 4 mM-Tris/HCl, pH 7.8 at 22°C, containing 4 mM-2-mercaptoethanol, 4 mM-MgCl₂, 0.8 mM-EDTA (tetrasodium salt), 160 mM-(NH₄)₂SO₄ and 20% (v/v) glycerol. Protein concentration was 0.2 mg/ml, determined by the method of Lowry *et al.* (1951), with bovine plasma albumin (fraction V; Armour Pharmaceutical, Chicago, IL, U.S.A.) as standard.

Enzyme assay

Standard reaction mixtures (0.1 ml) contained 0.1 M-Tris/HCl (pH 7.6 at 22°C), 4 mM-2-mercaptoethanol, 0.4 mM-[8-¹⁴C]GTP (approx. 1 Ci/mol), 0.5 mg of bovine serum albumin/ml, 1 mM-phosphoenolpyruvate, 18.2 μ g of pyruvate kinase/ml, 2 A₂₇₀ units of poly(C)/ml, 10 mM-MnCl₂ and 20 μ g of phage-Q β RNA polymerase/ml. Final (NH₄)₂SO₄ concentration was 25 mM. With poly(U₂,C) as template (150 μ g/ml), 1 mM-ATP and 1 mM-MnCl₂ were used. Details of individual experiments are contained in Figure legends. Reactions were run for 20 min at 32°C and stopped by pipetting 80 μ l on to 2.5 cm Whatman 3 MM filters. Filters were dried for 30 s under warm air from a hair-dryer, plunged into cold 5% (w/v) trichloroacetic acid/4 mM-sodium pyrophosphate and washed as described by Stout & Mans (1967). Retained radioactivity was determined by counting dried filters in a toluene-based scintillation fluid containing 0.5% (w/v) 2,5-diphenyl-oxazole and 0.05% (w/v) 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl)benzene.

I₅₀ values, i.e. compound concentration giving 50% inhibition of GMP incorporation, were determined by preincubating enzyme and inhibitor for 60 min at 0°C in 0.05 ml volume. A mixture of the remaining components was added to start the reaction.

Preincubation experiments

To determine the effects of GTP, MnCl₂ and template on inhibition, a reaction mixture (0.06 ml) lacking the above components, enzyme and inhibitor was made up. The components to be studied were

added, then inhibitor and then enzyme to give 0.08 ml, and the final mixture was preincubated for 45 min at 0°C. Missing components were then added to start the reaction, which was run for 20 min at 32°C. Final volume was 0.1 ml.

Calculations

I₅₀ values were determined from a linear regression of percentage inhibition on log(concentration), and 95% confidence limits were calculated for single determinations as described by Snedecor & Cochran (1967).

Reagents

[8-¹⁴C]GTP (tetrasodium salt) (50.1 Ci/mol) was obtained from New England Nuclear (Boston, MA, U.S.A.). Nucleotides were from P & L Laboratories (Milwaukee, WI, U.S.A.). Poly(C) [potassium salt; s₂₀ in 50 mM-sodium phosphate, pH 7.0 (containing 0.1 M-NaCl) of 5.2-5.7 S] and poly(U₂,C) (potassium salt) were purchased from Miles (Elkhart, IN, U.S.A.) and Boehringer-Mannheim (New York, NY, U.S.A.) respectively. Rabbit muscle pyruvate kinase in 3 M-(NH₄)₂SO₄ from Calbiochem (San Diego, CA, U.S.A.) had a specific activity of 494 units/mg; 1 unit represents the production of 1 μ mol of pyruvate/min. All other chemicals were of reagent grade.

Results

Characterization of poly(C)- and poly(U₂,C)-dependent reactions

The poly(C)- and poly(U₂,C)-dependent reactions are characterized with respect to time and protein-dependence in Fig. 1. Although poly(U₂,C)-directed synthesis ceases after 20 min, the poly(C)-directed reaction continues at a falling rate even beyond 30 min, up to at least 60 min, as shown by Mitsunari & Hori (1973). An initial linear incorporation for 10 min is seen with both templates. Product increases linearly with protein up to at least 12 μ g/ml.

Titration of both templates with Mn²⁺ and Mg²⁺ (Figs. 2 and 3) give linear double-reciprocal plots and reveal absolute dependence on added nucleic acid for GMP incorporation. Reactions with Mn²⁺ run 3-5 times faster than those with Mg²⁺, and K_m values for template, expressed as nucleotide concentration, range from 10 to 40 μ M (Table 1). Mitsunari & Hori (1973), in their study of the poly(C)-directed reaction, also found a higher reaction rate with Mn²⁺, and they reported K_m values of 12 and 17 μ M for Mg²⁺ and Mn²⁺ respectively.

The metal-ion titrations for the two reactions (Fig. 4) show some differences: with poly(U₂,C) a sharp

Fig. 1. Kinetics of synthesis (a) and protein-dependence (b) for phage-Q β RNA polymerase reactions

For kinetics with poly(C) (●) a 0.25 ml reaction mixture was prepared as described in the Materials and Methods section with 16 μg of polymerase/ml. For kinetics with poly(U₂C) (○) a similar mixture was used, but with 2 mM-MnCl₂, 1 mM-ATP, 150 μg of poly(U₂C)/ml instead of poly(C), and 14 μg of polymerase/ml. Protein-dependence curves for poly(U₂C) (○) and poly(C) (●) were determined in 20 min incubations under the same conditions as the kinetic studies. GMP incorporation is reported as nmol/20 min per 0.1 ml.

Fig. 2. Double-reciprocal plots for the effect of poly(C) concentration on the activity of phage-Q β RNA polymerase with 10 mM-Mg²⁺ (a) and 10 mM-Mn²⁺ (b)

Reaction mixtures (0.1 ml) were prepared as described in Fig. 1 with 20 μg of polymerase/ml and various concentrations of template. Incubation was for 20 min at 32°C. Inserts show original data.

Mn²⁺ optimum is observed at 1 mM, but with poly(C) the optimum is at 10 mM-Mn²⁺. With Mg²⁺ the optima are broad and similar, i.e. 10 mM. In poly(C)-directed synthesis a combination of equimolar Mn²⁺

and Mg²⁺ gives the same incorporation as Mn²⁺ alone (results not shown).

The ATP requirement for poly(U₂C)-directed synthesis is satisfied below 0.5 mM with either metal

Fig. 3. Double-reciprocal plots for the effect of poly(U_2C) concentration on the activity of phage- $Q\beta$ RNA polymerase with 10 mM- Mg^{2+} (a) and 2.5 mM- Mn^{2+} (b)

Reaction mixtures were prepared as described in Fig. 1 with 13 μ g of polymerase/ml and various concentrations of template. Incubation was for 20 min at 32°C. Inserts show original data.

Table 1. Comparison of apparent template K_m values and relative rates of reaction with Mg^{2+} and Mn^{2+} for phage- $Q\beta$ RNA polymerase with poly(C) or poly(U_2C) as template

Reactions were run under optimum conditions for determination of the rate ratio. Apparent K_m values, expressed as nucleotide concentration, are derived from Figs. 2 and 3.

Template	Rate ratio Mn^{2+}/Mg^{2+}	Template K_m (μ M)	
		Mg^{2+}	Mn^{2+}
Poly(U_2C)	4.43	31	46
Poly(C)	3.33	26	9.2

ion. Fig. 5(a) shows results with Mg^{2+} ; results with Mn^{2+} (not shown) are similar. The GTP optimum with Mg^{2+} lies at or above 0.5 mM and its titration gives a non-linear double-reciprocal plot (Fig. 5b). When $1/[S]^2$ is plotted against $1/v$, however, a straight line is observed (results not shown), suggesting the involvement of two GTP-binding sites in the poly- (U_2C) -dependent reaction. Similar kinetics were seen for the poly(C)-dependent reaction of bacteriophage-f2 replicase (Fedoroff & Zinder, 1972). Mitsunari & Hori (1973) did not report a non-linear plot for phage- $Q\beta$ poly(C)-dependent RNA polymerase, but Hori (1974) has shown that there are two GTP-binding sites on this enzyme.

Inhibition by ortho- and pyro-phosphate

Effects of metal ion and template on concentrations of P_i and PP_i giving 50% inhibition of phage- $Q\beta$

RNA polymerase depend both on the template and on the metal ion (Table 2). Mn^{2+} promotes significantly ($P < 0.05$) greater inhibition than Mg^{2+} , except for P_i inhibition of the poly(U_2C)-dependent reaction, where there is no significant metal effect. Overall, PP_i is an inhibitor of phage- $Q\beta$ RNA polymerase that is relatively insensitive to the chosen combination of template and metal ion. I_{50} values for PP_i range from 0.241 mM to 1.82 mM, less than 10-fold. P_i , on the other hand, is most potent with Mn^{2+} and poly(C); the I_{50} increases by a factor of 10 or more if either Mg^{2+} or poly(U_2C) is substituted.

Preincubation experiments

The two templates and $MnCl_2$ were further examined and the effect of GTP on inhibition was investigated by including combinations of these reagents in a preincubation period along with enzyme, compound and other reaction components. Preincubation was on ice for 45 min in 0.08 ml. Reaction mixtures were completed by addition of missing components and incubated for 20 min at 32°C. Since preliminary work indicated that template alone had little effect on inhibition (see Table 3), we found an appropriate preincubation time by keeping a reaction mixture with all components except template on ice for various times before initiating synthesis with poly(C). No change in percentage inhibition occurs with either P_i or PP_i beyond 15 min preincubation (results not shown).

Inhibition by PP_i was largely unaffected by the presence or absence of GTP, Mn^{2+} or template,

Fig. 4. Dependence of phage-Q β RNA polymerase on Mn^{2+} (○) and Mg^{2+} (●) in the poly(U_2C)- (a) and poly(C)-dependent (b) reactions

For reactions with poly(U_2C) the template concentration was 5 μ g/ml with 12.8 μ g of polymerase/ml. Incubation was for 20 min at 32°C. All other conditions were as described in Fig. 1.

Fig. 5. Dependence of phage-Q β RNA polymerase on ATP (a) and GTP (b) with poly(U_2C) as template

Reactions were run with 10 mM-MgCl $_2$, 5 μ g of poly(U_2C)/ml and 13 μ g of polymerase/ml. For the ATP study (a), GTP concentration was 0.4 mM. For the GTP study (b), ATP concentration was 1 mM. Other conditions were as described in Fig. 1. Original data are shown as an insert in (b).

alone (Table 3) or in combination (Table 4). A suggestion of a protective effect of Mn^{2+} alone or of the Mn^{2+} -template combination is seen in the poly(C)-dependent reaction. The lack of major effects on PP $_i$ inhibition is in accord with the small

range of I_{50} values for PP $_i$ shown in Table 2. In contrast, inhibition of both reactions by P $_i$ is significantly accentuated by GTP in the preincubation step (Table 3). Combinations of GTP with Mn^{2+} or template also sensitize the enzyme to inhibition by P $_i$

Table 2. *Inhibition of phage-Q β RNA polymerase by P₁ and PP₁*

Inhibition is given as the I₅₀, the concentration of compound giving 50% inhibition of GMP incorporation. Values in parentheses are 95% confidence limits.

Template	I ₅₀ (mM)			
	P ₁		PP ₁	
	Mg ²⁺	Mn ²⁺	Mg ²⁺	Mn ²⁺
Poly(U ₂ ,C)	44.5 (24.2-91.1)	72.3 (26.2-452)	1.82 (1.54-2.15)	0.241 (0.158-0.367)
Poly(C)	62.7 (48.0-84.1)	0.470 (0.168-1.14)	3.44 (2.71-4.40)	0.801 (0.301-2.06)

Table 3. *Effect of preincubation with GTP, Mn²⁺, poly(C) and poly(U₂,C) on inhibition of phage-Q β RNA polymerase by P₁ and PP₁*

Data shown are means of two independent experiments for poly(C) and are from a single experiment for poly(U₂,C). Final concentrations were 0.32 mM-P₁ and 2.0 mM-PP₁.

Addition to preincubation mixture	Inhibition (%)			
	P ₁		PP ₁	
	Poly(C)	Poly-(U ₂ ,C)	Poly(C)	Poly-(U ₂ ,C)
None	58	13	66	57
GTP	83	42	66	70
Mn ²⁺	0	19	51*	73
Template	30	27	66	70

* Value from a single experiment.

Table 4. *Effect of preincubation with GTP, Mn²⁺, poly(C) and poly(U₂,C) in combinations on inhibition of phage-Q β RNA polymerase by P₁ and PP₁*

Conditions were as described in Table 3.

Addition to preincubation mixture	Inhibition (%)			
	P ₁		PP ₁	
	Poly(C)	Poly-(U ₂ ,C)	Poly(C)	Poly-(U ₂ ,C)
None	58	13	66	57
GTP, Mn ²⁺	46	40	63	60
GTP, template	50	73	78	71
Mn ²⁺ , template	26	0	48	57

in the poly(U₂,C)-directed reaction (Table 4). Analogous to the results with PP₁, Mn²⁺, alone or in combination, protects the enzyme in poly(C)-directed reactions.

It is noted that P₁-inhibited reactions with poly(C) as template react to preincubation conditions differently from those with poly(U₂,C). This is most evident in response to Mn²⁺ and template, which fail

to protect the poly(U₂,C)-directed reaction, whereas the same components completely or partially prevent inhibition of the poly(C)-directed reaction (Table 3). In combination, however, Mn²⁺ and template afford partial or complete protection from P₁ inhibition to both enzyme systems (Table 4).

Effect of GTP concentration on inhibition of poly(C)-directed reaction

The marked sensitization of both reactions to P₁ inhibition brought about by GTP led us to examine the effect in more detail for poly(C)-directed synthesis. Various concentrations of GTP and either P₁ (Fig. 6a) or PP₁ (Fig. 6b) were preincubated with enzyme on ice for 60 min. Mixtures were then completed and incorporation at 32°C for 4 min was determined. GTP promotes inhibition at higher concentrations of the nucleotide but prevents inhibition when it is used at lower concentrations. Indeed at the lowest concentrations of inhibitor used (103 μ M-P₁ and 257 μ M-PP₁) there is stimulation of incorporation at low GTP concentrations compared with control reactions run at the same GTP concentration but without inhibitor. In control reactions GMP incorporation was the same whether GTP was added either during incubation only or during both preincubation and incubation (results not shown).

Discussion

These experiments document the complexity of inhibition of phage-Q β RNA polymerase. This complexity is evident even though we have chosen two relatively uncomplicated reactions and two simple and related inhibitors. Inhibitory potency is shown to be a function of template, metal ion and substrate. These facts emphasize the hazards of using inhibition data from a polymerase system for structure-activity analysis without comparing inhibitors under more than one condition.

The influence of template on inhibition is more pronounced with P₁ than with PP₁. With Mn²⁺ as cofactor, for example, P₁ is 150 times more potent an

Fig. 6. Inhibition of poly(C)-dependent phage-Q β RNA polymerase by P_i (a) and PP_i (b) as a function of GTP concentration. Preincubation mixtures (55 μ l) contained 2.95 μ g of polymerase, various concentrations of GTP, and either P_i (○, 206 μ M; ●, 103 μ M) or PP_i (○, 1.03 mM; ●, 0.51 mM; □, 0.257 mM). After 60 min at 0°C, mixtures were completed by addition of other components (45 μ l), including $MnCl_2$, to give final concentrations described in the Materials and Methods section. Inhibitor concentrations refer to final incubation conditions. Incorporation was determined after 4 min of synthesis.

inhibitor of the poly(C)-directed reaction than of the poly(U₂C)-directed reaction. Template-based differences are also apparent from results of preincubation experiments. Both Mn^{2+} and template by themselves afford protection from P_i inhibition only to the poly(C)-dependent reaction. One possible basis for these differences may be the use of different sites on the enzyme for processing each template. Kondo & Weissmann (1972) have drawn a distinction between the site on phage-Q β RNA polymerase at which RNA first binds and the site for initiation of synthesis, based on preferential inhibition of the latter by poly(ethylenesulphonate). Hori (1973) has presented kinetic evidence that the phage-Q β RNA- and poly(C)-binding sites are distinct on phage-Q β RNA polymerase. Kamen *et al.* (1972) showed that phage-Q β replicase lacking its 70000-mol.wt. subunit can still use poly(C) as template, but not phage-Q β viral RNA. If poly(U₂C) is sufficiently RNA-like to use the RNA site(s), then inhibition by P_i and PP_i may reflect binding of inhibitor to different sites. Alternatively a single inhibitor-binding site could affect the two template-binding sites differently.

Mn^{2+} , in general, promotes the potency of P_i and PP_i when it is added after enzyme and inhibitor have been preincubated (Table 2), but it protects when added before the inhibitors (Table 3). We rule out formation of metal-inhibitor complex as the basis

for these effects, since (1) both protection and sensitization occur under conditions where complex is present and (2) GTP is able to restore inhibition with metal ion present (Table 4) when complex is still available. One model to explain the metal ion effect on inhibition by P_i would postulate that metal ion interacts with polymerase to change to affinity of sites on the enzyme that bind template. Senear & Steitz (1976) have shown that the interaction of RNA with polymerase subunit S1 (largest) is modulated by Mg^{2+} . Metal ion decreases the yield of protected RNA fragments from ribonuclease digests of subunit-S1-RNA complexes. Further suggestion of a metal-ion-mediated change in enzyme-template interaction comes from the requirement for Mg^{2+} or Mn^{2+} to initiate synthesis after a non-dissociating complex is formed (Fig. 4, and Silverman, 1973). If metal ion is given a chance to act in the absence of P_i , it may tighten binding of template to enzyme, exclude inhibitor, and have the protective effect shown. If P_i is present, however, metal ion strengthens inhibitor binding to the enzyme at the expense of template. Consistent with this model is the pronounced protective effect of Mn^{2+} and template together on P_i inhibition (Table 4).

Perhaps the most obscure effect on inhibition by P_i and PP_i is that produced by GTP. The substrate both protects at low concentrations and sensitizes

at high concentrations. Silverman (1973) has shown that GTP is necessary to form a non-dissociating complex between phage-Q β RNA polymerase and either phage-Q β viral RNA or poly(C). Apparently this complex is sensitive to inhibition by P_i and PP_i (Table 4). As with template, the possibility exists that the effect of GTP on inhibition is related to the existence of two binding sites on phage-Q β RNA polymerase. Hori (1974) found, on the basis of inhibition by a thiol-blocking reagent, that this enzyme contains both a unique GTP-binding site and a second GTP-binding site shared by the other nucleoside triphosphates. Fedoroff & Zinder (1972) concluded, on the basis of the second-order dependence of reaction rate on GTP concentration, that the related phage-f2 replicase has two substrate-binding sites. We show here that phage-Q β replicase shows the same second-order dependence for the poly(U₂C)-dependent reaction (Fig. 5b). The role of GTP may involve binding at low concentration to one site, which prevents inhibition by P_i, and binding at high concentration to a second site of lower affinity, which potentiates inhibition by P_i.

It should be noted that the effects of Mn²⁺, GTP and template must be mediated through relatively stable complexes, since our experiments involved observing an effect of preincubation conditions after 20 min of synthesis, during which time all mixtures had been completed and were identical. Silverman (1973) found that the non-dissociating complex of RNA and replicase was stable even at 37°C for at least 24 min. Senear & Steitz (1976) report that a complex between phage-Q β RNA and Host Factor I, a host protein required for phage replication, is very stable at 0°C and decays only slowly at 25°C. The subunit-S1-RNA complex shows stability at 0°C. Thus our findings may be an extension of the reported stability of template-enzyme complexes to metal ion-enzyme and substrate-enzyme associations.

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