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The mechanism of pyrophosphorolysis of RNA by RNA polymerase

Endowment of RNA polymerase with artificial exonuclease activity

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1. DNA-directed RNA polymerase from *Escherichia coli* can break down RNA by catalysing the reverse of the reaction:

$$NTP + (RNA)_n = (RNA)_{n+1} + PP_i$$

where n indicates the number of nucleotide residues in the RNA molecule, to yield nucleoside triphosphates. This reaction requires the ternary complex of the polymerase with template DNA and the RNA that it has synthesized. 2. It is now shown that methylenebis(arsonic acid) [$CH_2(AsO_3H_2)_2$], arsonomethylphosphonic acid ($H_2O_3As–CH_2–PO_3H_2$) and arsonoacetic acid ($H_2O_3As–CH_2–CO_2H$) can replace pyrophosphate in this reaction. 3. When they do so, the low-M_r products of the reaction prove to be nucleoside 5′-phosphates, so that the arsenical compounds endow the polymerase with an artificial exonuclease activity, an effect previously found by Rozovskaya, Chenchik, Tarusova, Bibilashvili & Khomutov [(1981) *Mol. Biol. (Moscow)* **15**, 636–652] for phosphonoacetic acid ($H_2O_3P–CH_2–CO_2H$). This is explained by instability of the analogues of nucleoside triphosphates believed to be the initial products. 4. Specificity of recognition of pyrophosphate is discussed in terms of the sites, β and γ, for the $–PO_3H_2$ groups of pyrophosphate that will yield P-β and P-γ of the nascent nucleoside triphosphate. Site γ can accept $–AsO_3H_2$ in place of $–PO_3H_2$, but less well; site β can accept both, and also $–CO_2H$. 5. We suggest that partial transfer of an Mg^{2+} ion from the attacking pyrophosphate to the phosphate of the internucleotide bond of the RNA may increase the nucleophilic reactivity of the pyrophosphate and the electrophilicity of the diester, so that the reaction is assisted.

Rozovskaya *et al.* (1981*a*,*b*) showed that DNA-directed RNA polymerase (EC 2.7.7.6) from *Escherichia coli*, when in a ternary complex with template DNA and with the RNA that it has synthesized, can catalyse pyrophosphorolysis of this RNA. Nucleoside triphosphates must be absent, and Mg^{2+} ions are required. They also studied the substrate specificity of the reaction for PP_i (Table 1); one of its analogues they studied, phosphonoacetic acid, proved to be unusual, in that nucleoside monophosphates were produced rather than triphosphates or their analogues; in other words, the overall reaction of the polymerase had become that of an exonuclease. We now extend their findings by examining PP_i analogues that contain the arsono group, $–AsO_3H_2$, as these might be expected to act similarly if substrates for the enzyme. Part of the interest of discovering what analogues of PP_i can replace it in pyrophosphorolysis is that action on a DNA polymerase can explain the ability of some of them to inhibit viral replication (Mao *et al.*, 1975; Bolden *et al.*, 1975; Leinbach *et al.*, 1976; Alloudeen & Bertino, 1978).

Materials and methods

The kinetics of RNA degradation from the native ternary complex with D111 T7 DNA and RNA polymerase in the presence of PP_i and its analogues was analysed by electrophoresis of the

RNA in polyacrylamide gel and by chromatography of the low-M_r products on polyethyleneimine–cellulose (Rozovskaya *et al.*, 1981*a*,*b*). [^{32}P]-RNA was synthesized from the A1 promoter of D111 T7 DNA for 10min at 23°C in the presence of 100μM-CpA and 2.5μM of each of the four nucleoside triphosphates, one of them being labelled with a specific radioactivity of 100–400Ci/mmol. The resulting ternary complex of RNA polymerase, D111 DNA and nascent RNA was purified from NTPs by gel filtration on A1.5m agarose. For pyrophosphorolysis of the RNA, the ternary complex was incubated with 1mM-PP_i or one of its analogues in the presence of Mg^{2+}. Details, together with the purification and properties of the RNA polymerase, the D111 T7 DNA and other materials, are given by Rozovskaya *et al.* (1981*a*,*b*). The conditions of synthesis and of pyrophosphorolysis were such that equilibrium between these processes was never approached in our experiments. In the synthesis with 2μM of each NTP for 8–10min at 22°C, about half the radioactivity added as [α-^{32}P]CTP was incorporated; the low rate of pyrophosphorolysis meant that low-M_r products, whether with PP_i or with one of its analogues, reached a concentration of only about 0.1μM.

Methylenebis(arsonic acid) was prepared by the method of Popp (1949): As_2O_3 was treated with $AlCl_3$ and acetyl chloride, and then with $SOCl_2$, to form methylenebis(dichloroarsine) [$CH_2(AsCl_2)_2$]; this was purified by distillation and was treated with H_2O_2 to give the $CH_2(AsO_3H_2)_2$. The acid obtained was purified by adjusting a solution in water to pH6 with cyclohexylamine, evaporating to dryness, and crystallizing its salt with two molecules of cyclohexylamine from methanol on addition of diethyl ether. This salt gave a single spot, with a mobility slightly greater than that of arsenate, on paper electrophoresis at pH6.5; details of the system are given by Adams *et al.* (1983), and included detecting spots by their ability to bind Fe^{3+} (Wade & Morgan, 1953). Elemental analysis gave C, 32.5; H, 6.85; N, 5.7%; $CH_6As_2O_6,H_2O,2C_6H_{13}N$ requires C, 32.5; H, 7.1, N, 5.8%, but direct evidence for hydration was not obtained because the material lost cyclohexylamine on heating. Arsonomethylphosphonic acid was prepared by treating chloromethylphosphonic acid with alkaline arsenite, and was crystallized as its salt with two molecules of cyclohexylamine (Webster *et al.*, 1978). This material was characterized by X-ray crystallography (Falvello *et al.*, 1977). Arsonoacetic acid was prepared by treating chloroacetic acid with alkaline arsenite, and the product was isolated as its barium salt (Palmer, 1925); this salt was dissolved by stirring with a sulphonated polystyrene resin (Duolite 225 SRC 15) in its H^+ form, and the suspension was filtered through a bed of the same resin. The solution was concentrated to dryness with the bath at 30°C, when the free acid crystallized. Elemental analysis gave C, 12.9; H, 2.7%; $C_2H_5AsO_5$ requires C, 13.0; H, 2.7%.

Results

Rozovskaya *et al.* (1981*a*) showed that addition of PP_i to the ternary complex of the RNA polymerase of *E. coli* with DNA and RNA leads to a processive pyrophosphorolysis of the RNA.

Table 1. *Analogues of PP_i (i.e. of diphosphate) as substrates in the pyrophosphorolysis of RNA catalysed by DNA-dependent RNA polymerase from E. coli*

The symbol –*P* means –P(O)$(OH)_2$ (and its ionized forms) when it is joined to one other symbol, whereas –*P*– means –P(O)(OH)– (and its ionized form) when it is joined to two other symbols, as recommended by the IUPAC–IUB Commission on Biochemical Nomenclature (1966, 1978). Key to references: I, Rozovskaya *et al.* (1981*b*); II, the present work.

Compound	Substrate action	Low-M_r products	Reference
P–CH_2–*P*	+	Nuc–O–*P*–O–*P*–CH_2–*P*	I
P–NH–*P*	+	Nuc–O–*P*–O–*P*–NH–*P*	I
P–CH_2–CO_2H	+	Nuc–O–*P*	I
H–*P*–CH_2–*P*	–		I
P–CH_2–*P*–CH_3	–		I
H–*P*–CH_2–*P*–CH_3	–		I
H–*P*–CH_2–CO_2H	–		I
HO_2C–CH_2–CO_2H	–		I
P–O–*P*–O–*P*	+	Nuc–O–*P*–O–*P*–O–*P*+P_i	I
P–O–*P*–CH_2–*P*	+	Nuc–O–*P*–O–*P*–CH_2–*P*+P_i	I
P–CH_2–*P*–CH_2–*P*	–		I
P–CH_2–AsO_3H_2	+	Nuc–O–*P*	II
H_2O_3As–CH_2–AsO_3H_2	+	Nuc–O–*P*	II
H_2O_3As–CH_2–CO_2H	+	Nuc–O–*P*	II

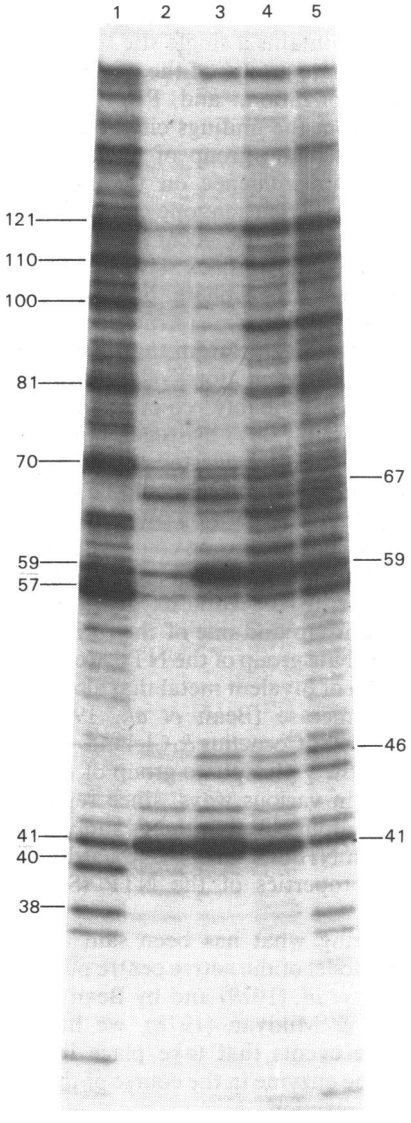


Fig. 1. *Electrophoresis of RNA before and after pyrophosphorolysis*

RNA was isolated from the ternary complex with RNA polymerase and template DNA after pyrophosphorolysis with 1 mM-PP_i or -analogue for 12h at 22°C, and was submitted to electrophoresis. Tracks: 1, not incubated; 2, *P*–O–*P*; 3, *P*–CH_2–AsO_3H_2; 4, H_2O_3As-CH_2–AsO_3H_2; 5, H_2O_3As-CH_2–CO_2H. The numbers at the sides of the electrophoretograms indicate the number of nucleotide residues in each species, including initiating dinucleoside phosphate from which numbering was started.

Electrophoretic analysis of the RNA synthesized from the A1 promoter showed that, under the conditions used, RNA of length 20–150 nucleotide residues was formed. The relative content of RNA

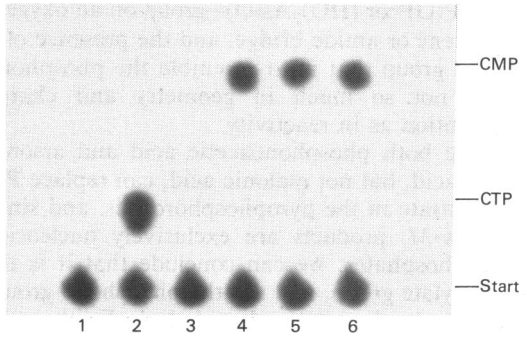


Fig. 2. *Chromatography of low-M_r products of RNA pyrophosphorolysis*

RNA, labelled with [α-^{32}P]CTP during enzyme-catalysed synthesis, was incubated, in the form of its ternary complex with RNA polymerase and template DNA, with 1 mM-PP_i or -analogue for 12h at 22°C. The products were chromatographed on polyethyleneimine–cellulose, and radioautographs were made of the plates. Tracks: 1, no incubation; 2, *P*–O–*P*; 3, HO_2C–CH_2–CO_2H; 4, H_2O_3As–CH_2–CO_2H; 5, H_2O_3As–CH_2–*P*; 6, H_2O_3As–CH_2–AsO_3H_2.

of any given length was proportional to the intensity of the corresponding band. The substrate properties of PP_i and its analogues were assessed by their effects on the distribution of intensities of the RNA bands on the electrophorograms. Rozovskaya *et al.* (1981*b*) established that *P*–CH_2–*P*, *P*–NH–*P*, *P*–CH_2–CO_2H, *P*–O–*P*–O–*P* and *P*–O–*P*–CH_2–*P* (see Table 1) could replace PP_i in this reaction. We have now found that three arsenical analogues of PP_i, namely methylenebis(arsonic acid), arsonomethylphosphonic acid and arsonoacetic acid (Table 1), also have the same effect as PP_i on the pattern of band distribution, and are therefore also low-M_r substrates of RNA polymerase in the pyrophosphorolysis reaction (Fig. 1).

Fig. 2 shows the results of chromatographing the low-M_r products of the degradation of RNA, labelled during synthesis with [α-^{32}P]CTP, when treated with PP_i, malonic acid and three arsenical analogues. In the presence of these three analogues, the only low-M_r product of the degradation is [^{32}P]CMP. We note that neither 1 mM-P_i (results not shown) nor 1 mM-malonate (Fig. 2) proves to be a substrate for the pyrophosphorolysis.

Discussion

Mechanism of RNA polymerase

Comparison of the results obtained by Rozovskaya *et al.* (1981*b*) with the present ones (Table 1) allows us to specify what features are necessary in an analogue of PP_i for it to be a substrate for the pyrophosphorolysis reaction: the presence of one

$(HO)_2P(O)$– or $(HO)_2As(O)$– group on an oxygen, methylene or amide bridge, and the presence of a second group that must resemble the phosphono group not so much in geometry and charge distribution as in reactivity.

Since both phosphonoacetic acid and arsonoacetic acid, but not malonic acid, can replace PP_i as substrate in the pyrophosphorolysis, and since the low-M_r products are exclusively nucleoside monophosphates, we can conclude that it is the carboxylate group, and not the phosphono group, that attacks the internucleotide link. Further, the fact that arsonomethylphosphonic acid also replaces PP_i, and also forms only nucleoside monophosphates, similarly implies that the arsono group and not the phosphono group reacts.

The fact that the concentration of nucleoside monophosphates reaches only about 0.1 μM rules out the possibility that we saw no analogues of the type HO_2C–CH_2–*P*–O–*P*–O–Nuc or H_2O_3As–CH_2–*P*–O–*P*–O–Nuc merely because they entered the reverse (synthetic) reaction and established a steady-state concentration that might have been missed in comparison with that of the nucleoside monophosphates. We are therefore confident that neither *P*–CH_2–CO_2H nor *P*–CH_2–AsO_3H_2 reacts appreciably through its phosphono group.

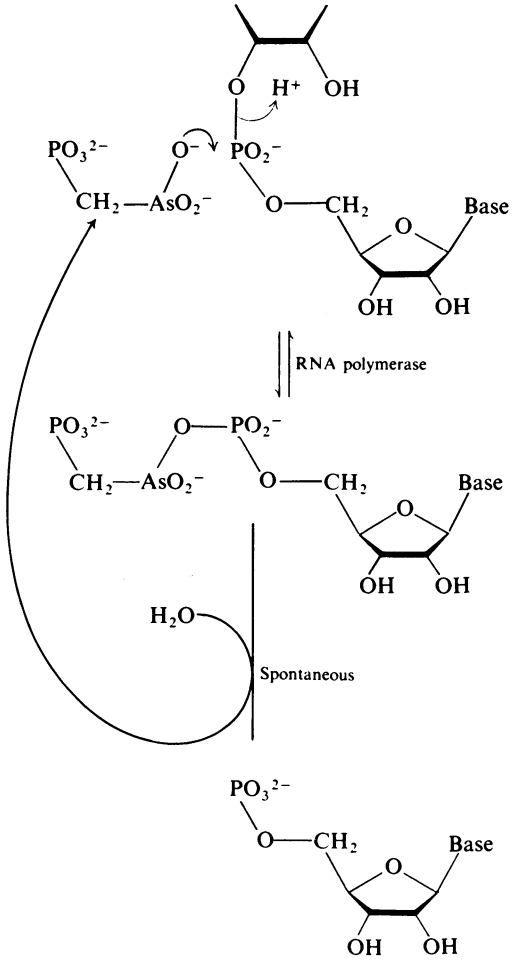
These conclusions, given that the arsono and phosphono groups have similar nucleophilic reactivity (Jencks & Carriuolo, 1959), imply that the PP_i-binding site of RNA polymerase has a definite asymmetry between its two parts: the γ-site, i.e. the site that binds the group containing P-γ of the nascent triphosphate, binds the phosphate group with great specificity, whereas the β-site holds the reacting part of the substrate so that it interacts with the phosphodiester group of the RNA and splits it. We note that the demands of the γ-site are strict; neither the carboxy group of *P*–CH_2–CO_2H nor the H–P(O)(OH)– or CH_3–P(O)(OH)– group of H–*P*–CH_2–*P*, H–*P*–CH_2–CO_2H or CH_3–*P*–CH_2–*P* can meet them. The fact that phophonoacetic acid (*P*–CH_2–CO_2H) attacks exclusively with its carboxy group suggests that the γ-site has an affinity for the phosphono group significantly greater (by more than 2–3 orders of magnitude) than for the carboxy group, and the fact that arsonomethylphosphonic acid attacks exclusively by its arsono group similarly implies that the γ-site binds the phosphono group much more strongly than it does the arsono group.

The demands made by RNA polymerase for an analogue of PP_i to be a substrate in the pyrophosphorolysis reaction are stricter than would have been expected from the substrate specificity of this enzyme in the elongation reaction (Rozovskaya *et al.*, 1981*b*, and references cited therein). Since pyrophosphorolysis is the reverse of elongation, the enzyme contains a single site that binds the β- and γ-phosphate groups of the nucleoside triphosphate in elongation, and PP_i in the reverse reaction. From the findings cited on modification of the triphosphate group of nucleoside triphosphates and its influence on their properties as substrates for elongation, one can suggest that the following interactions occur in the productive enzyme–substrate complex: (1) one of the oxygen atoms of the α-phosphate group of the NTP interacts with the enzyme (Armstrong *et al.*, 1979), possibly with the zinc atom that is tightly bound; (2) one of the oxygen atoms of the β-phosphate of the NTP is probably co-ordinated with the enzyme, possibly by hydrogen-bonding; Armstrong *et al.* (1979) suggested this to explain how both isomers of β-thioATP could be substrates, on the basis that competition by the enzyme with magnesium for this oxygen could force the magnesium to overcome its normal preference for oxygen over sulphur; (3) a second oxygen atom of the β-phosphate group and one of the oxygen atoms of the γ-phosphate group of the NTP are co-ordinated with the ion of bivalent metal that also binds to the RNA polymerase (Bean *et al.*, 1977; Stein & Mildvan, 1978; Cornelius & Cleland, 1978); (4) the fixation of the γ-phosphate group of the NTP can take place in various ways, since replacement of the oxygen atom between the β- and γ-phosphorus atoms by an NH or a CH_2 group hardly affects the substrate properties of the NTP (Simon *et al.*, 1965).

Considering what has been said already, and using the model of the active centre put forward by Armstrong *et al.* (1979) and by Bean *et al.* (1977) and Stein & Mildvan (1978), we have tried to picture the events that take place in the active centre of the enzyme in the course of the pyrophosphorolysis reaction.

The complex of PP_i (or one of its analogues) with an Mg^{2+} ion is likely to serve as the active form of the substrate. Such a complex binds to the enzyme so that one of its phosphono (or arsono) groups (in the case of unsymmetrical analogues, the unreplaced phosphono group) is fixed at the γ-site. This fixation apparently leads to the displacement of a water molecule from the co-ordination sphere of the magnesium by groups of the protein, and to the binding of this magnesium by one of the oxygen atoms on the phosphorus of the internucleotide link, i.e. the phosphorus atom that will be P-α of the nascent NTP. This binding has two effects: first, it increases the electrophilicity of this phosphorus atom: secondly, it diminishes electron attraction by the magnesium and thereby raises the nucleophilicity of the attacking PP_i (or analogue). Both effects facilitate the reaction. The interaction

between the magnesium and the phosphodiester group in the productive enzyme–substrate complex places the reagents in a conformation close to that of the transition state, in that the attacking and leaving groups occupy the apices of a trigonal bipyramid centred on a five-co-ordinate phosphorus atom. The NTP or analogue formed then dissociates from the complex in the form of its magnesium salt. The mechanism just described may largely be a reflection of the fact that magnesium binds to NTPs more strongly than to PP_i, so that the equilibrium of the polymerase reaction is less extreme than it would be for unbound species; hence it may not specifically stabilize the transition state in comparison with the products of pyrophosphorolysis.



Scheme 1. *Action of a PP_i analogue in conferring artificial exonuclease activity on RNA polymerase*

Artificial exonuclease activity

The conversion of the synthetic action of the polymerase into a degradative one depends on the use of a substrate analogue that enters into the catalysed reaction, but forms an unstable product (Scheme 1). This is like the classical use of arsenate in metabolic systems, explained by Braunstein (1931), and like the similar uncoupling of adenylate kinase by the two analogues of AMP in which the –O–*P* group is replaced by $-O-AsO_3H_2$ (Lagunas *et al.*, 1984) and by $-CH_2-AsO_3H_2$ (Adams *et al.*, 1984). In each case the analogue added is regenerated in the reaction and therefore acts catalytically.

Webster *et al.* (1978) cited several enzymes that cannot use arsonomethylphosphonic acid in place of PP_i; apparently DNA-directed RNA polymerase of *E. coli* is less specific.

The compounds now found to endow RNA polymerase with exonuclease action are those shown by Newton (1979) to diminish the biosynthesis of herpes-virus DNA in tissue culture, possibly also by acting as PP_i analogues.

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