Pyrophosphate-condensing activity linked to nucleic acid synthesis

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ABSTRACT

In some preparations of DNA dependent RNA polymerase a new enzymatic activity has been found which catalyzes the condensation of two pyrophosphate molecules, liberated in the process of RNA synthesis, to one molecule of orthophosphate and one molecule of Mg (or Mn) chelate complex with trimetaphosphate. This activity can also cooperate with DNA-polymerase, on condition that both enzymes originate from the same cells. These results point to two general conclusions. First, energy is conserved in the overall process of nucleic acid synthesis and turnover, so that the process does not require an energy influx from the cell's general resources. Second, the synthesis of nucleic acids is catalyzed by a complex enzyme system which contains at least two separate enzymes, one responsible for nucleic acid polymerization and the other for energy conservation via pyrophosphate condensation.

INTRODUCTION

It has been known for a long time that pyrophosphate is the product of many important metabolic processes continuously occurring in all cells; in particular, nucleic acid synthesis. However, pyrophosphate hardly ever accumulates in the cell and it is usually very difficult to detect it in cellular material. It appears that the general fate of pyrophosphate in vivo is to be metabolized as quickly as possible after being liberated (1).

The functional meaning of pyrophosphate removal is clear. The enthalpy and free energy changes in reactions of nucleic acid synthesis are small (2, 3). Were not the pyrophosphates immediately removed from the system, the equilibrium constants of these reactions should be close to 1, and approximately 50% of the NTP's could not be used for nucleic acid synthesis. The removal of pyrophosphate is required to shift the equilibrium of the reaction toward polymer synthesis. Moreover, L. Peller (4) has shown that if pyrophosphate is not removed, thermodynamics limits the number average degree of polymerization of RNA transcripts to substantially less than 100 nucleotide units of 33,000 daltons. Pyrophosphate removal raises the upper limit of
this average size by a factor of the order of $10^3$ and defers the approach to equilibrium.

However, in many cases where RNA transcription was performed in vitro and catalyzed by purified preparation of DNA-dependent RNA-polymerase, the pyrophosphate was not removed and remained intact in the reaction mixture. One might suppose that under such conditions the addition of inorganic pyrophosphatase would raise the yield of reaction and the average size of the products. However, using one of our preparations of RNA-polymerase, we found no difference either in the length distribution of newly formed nucleic acid chains or in reaction kinetics, whether the in vitro transcription was performed with or without the addition of inorganic pyrophosphatase.

In our former paper (2) we proposed that the pyrophosphate is not simply hydrolysed, but rather is removed from the system via another reaction in which the energy of the precursor's disrupted high energy bonds is stored in reaction products and can be used later by the cell. The aim of the present work was to investigate the ways of removal of pyrophosphates liberated in reactions of nucleic acid synthesis.

MATERIALS AND METHODS

Materials. Reactions of RNA transcription were carried out on SV-40 DNA, form I, kindly given to us by Dr. M. Oren from this Institute.

Two separate preparations of E. Coli DNA-dependent RNA polymerase were used in these experiments:

Preparation A was isolated and purified by Dr. A. Goldfarb from E. Coli, strain MRE 600 as described (5). Preparation B was isolated and purified by Dr. M. Zeevi (both from this Institute) from the same strain of E. Coli as described by Burgess and Jendrisak (6) and was further purified by affinity chromatography on a Blue Dextran Sepharose column (7, 8).

Both preparation A and B of RNA polymerase were examined by polyacrylamide gel electrophoresis as described (6).

The highly purified preparation B has given only four bands, which correspond to the known subunits of the enzyme (6). On the contrary the more crude preparation A has given many additional bands (data not presented).

DNA polymerase I (Kornberg's polymerase) from E. Coli, strain MRE 600 was received from Boehringer Mannheim GmbH, and T4 DNA polymerase - from P-L Biochemicals, Inc.

AMV RNA-dependent DNA polymerase (reverse transcriptase) was a gift from Dr. Beard from NIH.
Inorganic pyrophosphatase was purchased from Worthington. Unlabeled nucleotides, trimetaphosphate and triphosphate were obtained from Sigma. $^{32}$P labeled nucleotides, $^{32}$P labeled ATP, $^{32}$P labeled ortho- and pyrophosphate were received from Amersham. Other $^{32}$P labeled precursors (UTP, CTP, GTP and dCTP) were prepared in the following way: a reaction mixture of 100 μl containing 100 mM Tris-HCl, pH 8.1, 10 mM MgCl$_2$, 50 mM KCl, 4 mM β-mercaptoethanol, 2 mM sodium EDTA, 10 mM of one of NTP's or dCTP, 0.5 mCi of $^{32}$P ATP (10-20 Ci/mmol) and 5 μl of 1 mg/ml E. Coli kinase fraction was incubated for 10 minutes at 37°C, then heated for 3 minutes at 95°C and centrifuged to remove coagulated proteins. The supernatant was purified by thin layer chromatography on PEI cellulose using 0.3 N NH$_4$HCO$_3$ as running liquid. The spots of $^{32}$P-NTP or $^{32}$P-dCTP were cut out, labeled material was eluted with 0.5 N NH$_4$HCO$_3$, lyophilized and dissolved in H$_2$O.

Activated DNA for DNA polymerase reactions was prepared by limited digestion of "native" calf thymus DNA (Sigma) with pancreatic deoxyribonuclease as described (17). Globin mRNA was purified from reticulocytes of anemic mice as described (9).

Methods. RNA synthesis was performed at 30°C in 50 μl of reaction mixture containing: 40 mM Tris-HCl buffer, pH 7.9, 150 mM NaCl, 4 mM MgCl$_2$, 10 mM MnCl$_2$, 14 mM β-mercaptoethanol, 0.02 mg/ml SV-40 DNA, 0.25 mM of each UTP, GTP, CTP, 0.04 mM $^{32}$P ATP and 5 u/ml E. Coli RNA polymerase.

DNA synthesis was performed at 37°C in 50 μl of reaction mixture containing: 40 mM Tris-HCl, pH 7.9, 150 mM NaCl, 4 mM MgCl$_2$, 2 mM MnCl$_2$, 2 mM β-mercaptoethanol, 0.2 mg/ml of activated calf thymus DNA; 0.2 mM of each dATP, dGTP, TTP, 0.04 mM $^{32}$P dCTP and 5 u/ml of E. Coli DNA polymerase I or T4 DNA polymerase.

Reverse transcription was performed at 37°C in the same reaction mixture as DNA synthesis, using 0.1 mg/ml of globin mRNA and 0.02 mg/ml of oligo-dT (Collaborative Research, Inc.) as template-primer and 5 u/ml of reverse transcriptase as the catalysing enzyme.

The reaction products were separated by thin layer chromatography on PEI-cellulose plates (DC-Alufolien, Merck). Separation at neutral pH was made using 300 mM NH$_4$HCO$_3$. Two running liquids for two-dimensional chromatography were prepared from the following solutions:

Solution 1: 70 ml isopropanol + 100 ml H$_2$O.

Solution 2: 12.5 g trichloroacetic acid + 3.2 ml 25% ammonia sol. + H$_2$O to 100 ml.
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Solution 3: 20 ml 96% glacial acetic acid + 80 ml H$_2$O.
Solution 4: 7.5 g trichloroacetic acid + 80 ml 25% ammonia sol. + H$_2$O to 100 ml.
The acidic running liquid (pH 2) contained: 75 ml methyl alcohol + 20 ml solution 1 + 25 ml solution 2 + 6 ml solution 3. The alkaline running liquid (pH 11) contained: 67.5 ml methyl alcohol + 6 ml solution 3 + 50 ml solution 4.

Chromatograms were stained by the molybdate reagent after Stahl (10).

RESULTS

To elucidate the fate of pyrophosphate liberated in reactions of RNA transcription we have carried out these reactions in vitro using $\gamma ^{32}$P-ATP as the labeled precursor. Reaction products were separated by thin-layer chromatography using NH$_4$HCO$_3$ as the running liquid. The labeled products were located by autoradiography and the unlabeled ones by staining the plates with the molybdate reagent. $^{32}$P-labeled ortho- and pyrophosphate as well as unlabeled trimetaphosphate and triplyphosphate were used as markers.

Though both preparations of RNA polymerase used in these experiments were active in RNA synthesis, the by-products of their reactions were quite different. In reactions catalyzed by the preparation B the liberated pyrophosphate remained in the reaction mixture and no other labeled products could be detected (Fig. 1).

On the contrary, in reactions catalyzed by the preparation A pyrophosphate did not appear in the reaction mixture, but two different and new labeled products accumulate and appeared on the chromatograms as two separate spots I and II (Fig. 2). The same results were obtained with other $^{32}$P-labeled NTP's.

The product which accumulates in spot I can be identified immediately as the orthophosphate by comparison with the marker. It was quite natural to suppose that the other product is a triphosphate, and that the pyrophosphates have been removed via the enzyme catalyzed reaction:
**Figure 1:** Thin layer chromatographic separation of the products of RNA synthesis catalyzed by the preparation B of RNA polymerase with $\gamma^{32}\text{P}-\text{ATP}$ as the labeled precursor. 1 – markers, 2-5 – reaction products after 0, 10, 20, 30 minutes.

**Figure 2:** Thin layer chromatographic separation of the products of RNA synthesis catalyzed by the preparation A of RNA polymerase with $\gamma^{32}\text{P}-\text{ATP}$ as the labeled precursor. 1 – markers, 2-7 – reaction products after 0, 10, 20, 40, 90, 150 minutes.
\[(P \sim P) + (P \sim P) \xrightarrow{\delta} P_4 + (P \sim P \sim P) \quad (I)\]

For the sake of brevity we shall call the enzymatic activity which catalyzes this reaction the "pyrophosphate condensing" or "condensing" activity.

To be sure that these labeled products originate only from the pyrophosphate that was produced in the reactions of RNA synthesis, we repeated these experiments using the \(\alpha^{32}\)P-ATP instead of \(\gamma^{32}\)P-ATP used previously. In these experiments we were able to prove that almost the entire radioactivity of the labeled precursor could be recovered in TCA-precipitable RNA. When the reaction products were separated by thin-layer chromatography only two labeled spots could be seen on chromatograms: one bright spot at the origin attributable to RNA and a weak spot which could be identified as ADP by comparison with the marker. No traces of either free AMP or a triphosphate could be detected (data not presented).

Measurements of label distribution between RNA and ADP showed that only a small part of the precursors (approximately 5-8\%) undergo the triphosphatase reaction, in which the terminal orthophosphate is liberated. The bulk of precursor pool is involved in reactions of RNA synthesis, in which the pyrophosphates should be liberated. The absence of AMP and triphosphates in the reaction mixture shows that the labeled products we have obtained as spots I and II originate really from pyrophosphates liberated in reactions of RNA synthesis.

To be sure that RNA synthesis in these experiments was only DNA dependent, control experiments without DNA were performed. No RNA synthesis could be observed in absence of DNA.

Strong evidence in favor of our suggestion that reaction (I) is the way for pyrophosphate removal was obtained from measurements of label distribution between the products II and I. According to equation (I) the stoichiometric relationship of these products is 1:1, and the label must therefore be distributed between them in the proportion 3:1 (triphosphate: monophosphate), because each of four P atoms has the same probability of being labeled. To check out this conclusion five aliquots were taken from a reaction mixture at various times during the linear part of the reaction. The labeled products were separated and located as described above. The five pairs of spots I and II were scraped off of the chromatograms and their radioactivity was measured in a liquid scintillation counter. The ratio of radioactivity of substance II to that of the substance I was found to remain constant during the reaction time. The mean value of this ratio is 3:1,17 + 0,03.

This result is in excellent accord with the prediction of equation (I),
because the excess of I in the orthophosphate pool is fully accounted for by the previous finding that approximately 5-8% of all precursors undergo the NTP-ase reaction and give extra orthophosphate.

These data leave little if any doubt that product II is a triphosphate. However, its chemical identification presented some difficulties. It cannot be the trimetaphosphate, because under our conditions of chromatography it runs ahead of orthophosphate, whereas the marker (Na-trimetaphosphate) lags behind orthophosphate. Nor can it be tripolyphosphate, because $R_f$-value for the latter is smaller even than that of pyrophosphate.

It was, however, clear from the beginning that the final product we observe cannot be the primary product of reaction (I). Since the energy is conserved in this reaction (see Discussion), free energy changes are small, and the equilibrium constant of this reaction should be close to 1. Therefore, approximately 50% of the labelled pyrophosphate should remain in the reaction mixture, were not the primary products of condensing reaction (I) immediately modified to a form in which they become inaccessible to the enzyme. Since it is known that the presence of Mg and/or Mn ions is an absolute requirement for all reactions of nucleic acid synthesis, we suggested that our product II might be the Mg (or perhaps Mn) chelate of trimetaphosphate. This suggestions was substantiated by the following three experiments:

a) One could expect this chelate complex to become dissociated if the chromatography were performed at other values of pH, and this was borne out by experiment. Fig. 3 shows the results of a two-dimensional thin-layer chromatographic separation of the same products as in Fig. 2. The first (horizontal) separation was performed with the acidic running liquid (pH 2), the second (vertical) with the alkaline running liquid (pH 11). One can see that in acidic medium the marker (Na-trimetaphosphate) remains practically on the start line, whereas our product runs ahead of orthophosphate. In alkaline running liquid however $R_f$-values for our product and the marker are practically identical.

b) Commercial (Sigma) trimetaphosphate was boiled for several minutes in a solution containing 0.1 M MgCl$_2$ or MnCl$_2$. The only one possible product of this reaction would be a complex of Mg (or Mn) with trimetaphosphate. Thin-layer chromatography showed the reaction mixture to contain a product that coincided with our product II (Fig. 4).

c) Product II was eluted from the chromatogram with 0.5 M NH$_4$HCO$_3$, lyophylized, re-dissolved in water and applied to a DEAE Sephacel column.
Figure 3: Two-dimensional thin layer chromatographic separation of the same reaction products as in Fig. 2. 1 - origin, 2 - reaction product I (identified as orthophosphate by control experiment with markers), 3 - reaction product II, 4 - Na-trimetaphosphate (made visible by staining).

Figure 4: Thin layer chromatography of Na-trimetaphosphate boiled in 0.1 M MgCl₂ or MnCl₂. 1 - Na-trimetaphosphate, 2 - Na-trimetaphosphate boiled for 3 min. in H₂O, 3 - orthophosphate, 4 - Na-trimetaphosphate boiled for 3 min. in 0.1 M MgCl₂, 5 - Na-trimetaphosphate boiled for 3 min. in 0.1 M MnCl₂. Made visible by staining.
Fig. 5 shows that the charge of this product is approximately -1, in agreement with its supposed chemical makeup.

We can therefore conclude from the totality of the experimental evidence presented that there exists a specific pyrophosphate condensing activity in our preparation of RNA polymerase that removes the pyrophosphate liberated in reactions of RNA synthesis by converting two pyrophosphate molecules to one molecule of orthophosphate and one molecule of trimetaphosphate. That shifts the equilibrium of the RNA synthesizing reaction far to the right and allows transcription to proceed until the pool of precursors is exhausted. In this sense the pyrophosphate condensing activity is "linked" to RNA synthesis.

The next question we had to find an answer to was: are the liberated pyrophosphate molecules released into solution or do they remain bound to the enzyme system? To answer this question 32P-labeled pyrophosphate was added to the system in which all four NTP's remained unlabeled. When the reaction products were separated by thin-layer chromatography, only labeled orthophosphate could be seen on chromatograms and no traces of the second labeled product could be detected (Fig. 6).

Two conclusions follow from these results. First, the pyrophosphate condensing activity cannot act on free pyrophosphate in solution. One might argue the contrary: that the condensing activity can act on free pyrophosphate but is prevented from doing so, because the free pyrophosphate is

![Figure 5: Column chromatography of product II. Product II eluted from the chromatographic plate was analyzed by DEAE Sephacel chromatography as described under "Methods." Oligonucleotide markers of net charge -2 to -5 were determined by absorbance at 260nm. (••••)Absorbance at 260nm. (○○○) 32P.](image-url)
hydrolysed too rapidly by the pyrophosphatase present in our preparation. However, comparison of the kinetics of these two reactions (Fig. 2 and 6) shows that this is not the case.

Second, pyrophosphate molecules liberated in RNA transcription are protected against the pyrophosphatase activity. That means that they remain bound to the enzyme system containing the condensing activity. The whole process of pyrophosphate condensation proceeds in a unified enzyme complex. Even the addition of inorganic pyrophosphatase to the reactions with γ\(^{32}\)P-ATP did not change the results (data not presented).

After these facts had been established it was quite natural to suppose that perhaps the same pyrophosphate condensing activity might be responsible for the removal of pyrophosphate in DNA synthesis as well. To test this suggestion two kinds of DNA synthesizing reactions were performed.

1. Reverse transcription, i.e., DNA synthesis on RNA template and 2.
DNA synthesis on a DNA template. Template and enzymes used in these reactions are described in "Materials and Methods." $\gamma^{32}$P-labeled dCTP was used as the labeled precursor. Reaction products were separated by thin-layer chromatography and located as described above.

Though all the enzymes were active in DNA synthesis, the prophosphate condensing activity was lacking in all of them. Nothing but the labeled pyrophosphate could be detected. We then tried to restore the condensing activity by adding preparation A of RNA polymerase which contains this activity, to the reaction mixture. It was shown in control experiments that this enzyme does not interfere with dNTP's.

The pyrophosphatase activity was found in all three cases: orthophosphate instead of pyrophosphate appeared on chromatograms. In only one case, however, could the condensing activity also be detected. This occurred when both enzymes (DNA polymerase and RNA polymerase) were isolated from *E. Coli*. One can see in Fig. 7 a weak spot which corresponds to our product II, the Mg (or Mn) chelate of trimetaphosphate.

These results show that, first, the same condensing activity can interact...
with RNA- and DNA-polymerase and can catalyze the pyrophosphate condensing reaction in both cases. Second, this interaction seems to be species specific.

Since RNA polymerase is assumed to traverse the DNA template in steps of one nucleotide and since two pyrophosphate molecules are required for the condensation reaction (equation (I)), it is clear that the first pyrophosphate liberated must remain bound to the enzyme system and wait until the second is liberated. It is plausible to postulate the existence of specific pyrophosphate-binding sites in the enzyme system. Whether there is one binding site or two; and, if two, whether they are equivalent or non-equivalent; and if non-equivalent, whether the first pyrophosphate must be transferred from one site to the other - these are matters for future investigation.

There is, however, an alternative possibility that implies a different mechanism of polymerase action, namely, that the polymerase moves along the DNA template in steps of two nucleotides at a time. In this case two molecules of pyrophosphate would be liberated simultaneously. Condensation might take place simultaneously with pyrophosphate release or subsequently, but in either case there would be no first pyrophosphate molecule that would have to wait for the release of the second.

Experiments were carried out to distinguish between these alternative mechanisms; i.e., whether RNA polymerase elongates the growing RNA chain by one nucleotide at a time or by two. Oligo-A was synthesized on a poly-dT template and the reaction was stopped after a short time. The products were extracted and analyzed by electrophoresis in a 20% polyacrylamide - 7 M urea gel, using oligo-A of known size as markers. The results (data not presented) showed the reaction products to be a mixture of oligo-A's differing from each other by increments of one nucleotide. This eliminates the latter mechanism and shows that the RNA chain grows by the addition of one nucleotide at a time.

DISCUSSION AND CONCLUSIONS

It has been shown that the heat of hydrolysis is the same, 19 kcal/mol, for both cyclic trimetaphosphate and linear tripolyphosphate (11, 12). This value is equal to the energy of the two high energy bonds in two pyrophosphate molecules undergoing the condensation reaction and shows that the energy of these bonds is not lost for the organism as heat but rather is
stored in reaction products. This stored energy can be used later by the cell in many reactions of phosphate group transfer. In particular, this energy can be used to re-phosphorylate the "discharged" NDP - molecules to the level of NTP. An enzyme which catalyzes the reversible transfer of phosphate groups between polyphosphates and ADP was isolated and studied in Kornberg's laborabory (13, 14).

Salser, Janin and Levinthal (15) have found that the primary product of mRNA degradation in the cycle of mRNA turnover are nucleoside-diphosphates rather than nucleoside-monophosphates. They enter the NDP pool directly and not via the NMP's. The rationale for this finding was given in our previous paper (2). It has been shown that the energy of high energy bonds in the precursor which are disrupted in the process of RNA synthesis is stored in newly formed phosphodiester bonds. In reactions of RNA phosphoryl- olysis catalyzed by polynucleotide phosphorylase this stored energy is transferred back and restores the high energy bonds in NDP. These findings support the assumption that the degradation step in mRNA turnover is catalyzed by PNPase rather than by a nuclease. Recently Apirion (16) has shown that PNPase is at least one of the enzymes involved in the process of mRNA turnover.

By combining these results with data presented here we obtain evidence in favor of the general conclusion that energy is conserved in the whole process of RNA synthesis and turnover. The cycle of nucleic acid turnover is closed in energy as well as in matter. It does not require energy influx from the general resources of the cell.

Is the pyrophosphate condensing activity inherent to RNA-polymerase as such, or does it belong to a separate enzyme which acts in cooperation with the RNA-polymerase? The fact that this activity can be present in some preparations of RNA-polymerase and can be lacking in others makes the last assumption more plausible, but it does not give a definite answer to this question. It could be explained either by the assumption that the separate enzyme responsible for the condensing activity had been lost in process of RNA-polymerase purification, or by assumption that this activity was inherent to RNA-polymerase, but had been damaged during its purification.

However, the finding that the condensing activity linked to RNA-polymerase can cooperate with DNA-polymerase as well (on condition that both enzymes were isolated from the cells of the same species) argues strongly in favor of the suggestion that this activity belongs to a separate enzyme. That substantiates our general viewpoint that the synthesis of nucleic
acids in vivo is catalyzed by a complex enzyme system, which contains at least two enzymes: one responsible for RNA polymerization and the other responsible for pyrophosphate condensing and removal. These enzymes (and perhaps others too) act in a strictly coordinated manner.

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