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SHORT COMMUNICATION

INFLUENCE OF ALKALINE INORGANIC PYROPHOSPHATASE ON CHROMATIN-DIRECTED RNA POLYMERASE*

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Abstract—Alkaline inorganic pyrophosphatase from *Zea mays* removed pyrophosphate inhibition of RNA synthesis, on addition to *E. coli* RNA polymerase directed by chromatin from etiolated *Glycine max*. The ionic environment of the coupled reactions, even when optimized, caused reduced efficiency of both enzymes. Competition for Mg^{2+} by alkaline pyrophosphatase reduced RNA polymerase activity *in vitro*. The Mn^{2+} concentration optimal for RNA polymerase decreased the activity of alkaline inorganic pyrophosphatase by 80 per cent.

INTRODUCTION

BIOSYNTHETIC pathways for nucleic acids, coenzymes, proteins, carbohydrates, lipids and steroids have one or more synthetase reactions (pyrophosphorylases) which release inorganic pyrophosphate.[1,2] Kornberg[2] has proposed that these pathways are driven to completion by the rapid hydrolysis of pyrophosphate released by inorganic pyrophosphatase (E.C. 3.6.1.1.). This proposal has remained untested and its validity for carbohydrate transformations has been questioned.[3]

We investigated the effect of alkaline inorganic pyrophosphatase on *in vitro* chromatin-directed RNA synthesis since RNA synthesis *in vitro* is sensitive to inhibition by exogenous inorganic pyrophosphate.[4,5] Alkaline inorganic pyrophosphatase, which is active at the pH and Mg^{2+} level required by chromatin-directed RNA polymerase,[6] is the pyrophosphatase likely to relieve pyrophosphate inhibition of RNA synthesis leading to RNA formation after removal of one of the products of the reactions.[2]

RESULTS AND DISCUSSION

Preliminary experiments using soybean hypocotyl chromatin and partially purified alkaline inorganic pyrophosphatase from corn,[6] showed that ^3H–UMP incorporation by the endogenous RNA polymerase was too low for the accuracy and replication required. Therefore, the assays were augmented with purified *Escherichia coli* RNA polymerase.

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Figure 1 illustrates the inhibition of ^{3}H–UMP incorporation by 5 mM pyrophosphate. With the addition of 1·6 units alkaline inorganic pyrophosphatase, the pyrophosphate inhibition was relieved after a 6 min lag phase. The lag may be a reflection of the time required to remove the pyrophosphate in excess of the minimal, but variable, quantity causing complete inhibition. The reactions with continued pyrophosphate inhibition showed a net loss of ^{3}H–UMP incorporation possibly due to enzymatic hydrolysis of ^{3}H–RNA. The stimulation of RNA synthesis *in vitro* by addition of alkaline inorganic pyrophosphatase supports the view that inorganic pyrophosphatase can maintain biosynthesis by removal of an end-product. The high levels of alkaline inorganic pyrophosphatase in corn leaves which are capable of rapid photosynthesis[7,8] and the positive correlation of the pyrophosphatase activity with protein synthesis in bean leaf discs,[9] offer indirect evidence that the conclusions reached here apply *in vivo*.

In other experiments, we added 0·06 units alkaline inorganic pyrophosphatase to standard RNA polymerase assays. In some cases the time-course curves were similar in the

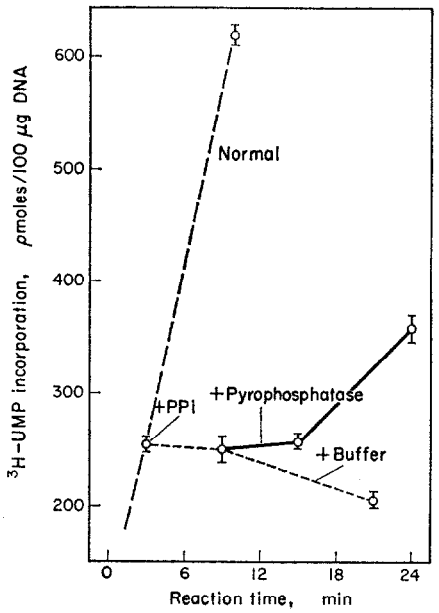

FIG. 1.

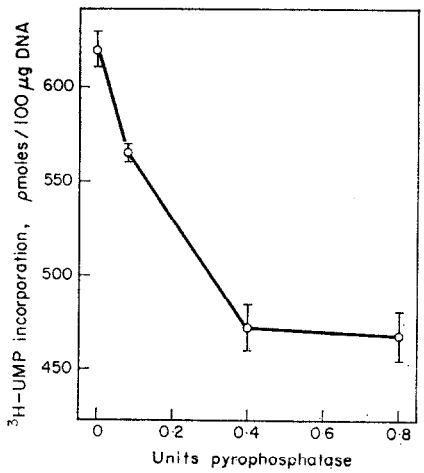

FIG. 2.

FIG. 1. THE EFFECT OF EXOGENOUS INORGANIC PYROPHOSPHATE AND ALKALINE INORGANIC PYROPHOSPHATASE ON CHROMATIN-DIRECTED RNA POLYMERASE. AFTER 3 min REACTION TIME IN STANDARD ASSAYS, 1 μmole PYROPHOSPHATE (pH 8·0) WAS ADDED TO SOME RNA POLYMERASE ASSAY TUBES. SIX min LATER, 1·6 UNITS ALKALINE INORGANIC PYROPHOSPHATASE (IN 50 mM Tris-HCl, pH 8·0, 1 mM $MgCl_2$) WAS ADDED TO SOME OF THESE ASSAYS, BUFFER WITHOUT INORGANIC PYROPHOSPHATASE TO THE OTHERS. MEAN AND STANDARD ERROR.

FIG. 2. THE EFFECT OF EXOGENOUS ALKALINE INORGANIC PYROPHOSPHATASE ON CHROMATIN-DIRECTED RNA POLYMERASE. STANDARD RNA POLYMERASE REACTIONS, RUN FOR 10 min, WERE AUGMENTED WITH INCREASING QUANTITIES OF ALKALINE INORGANIC PYROPHOSPHATASE. MEAN AND STANDARD ERROR.

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presence and absence of exogenous alkaline inorganic pyrophosphatase with maximal incorporation being reached after 15–20 min. In other runs, ^{3}H–UMP incorporation was considerably slower in the presence of alkaline inorganic pyrophosphatase but linear for as long as 40 min. When the quantity of alkaline inorganic pyrophosphatase was increased in 10 min incubations, ^{3}H–UMP incorporation decreased (Fig. 2).

The possibility that the small amount of ribonuclease activity accompanying the exogenous alkaline inorganic pyrophosphatase reduced ^{3}H–UMP incorporation was unlikely since the inhibition of incorporation was not proportional to added ribonuclease. Another possibility was that the incubation medium for RNA synthesis did not allow maximum rates of reaction simultaneously for both RNA polymerase and alkaline inorganic pyrophosphatase. This possibility was especially attractive when the complex ionic situation for the linked polymerase and pyrophosphatase reactions was considered. O'Brien *et al.*[4] showed that RNA polymerase was inhibited 94% in the absence of both Mg^{2+} and Mn^{2+} while omission of only Mg^{2+} caused 46% inhibition. *E. coli* RNA polymerase has similar Mg^{2+} and Mn^{2+} requirements for optimum activity (suppliers specifications). The Mn^{2+} concentration optimal for RNA polymerase activity reduces inorganic pyrophosphatase activity by 53%.[6,10] We found that the efficiency of alkaline inorganic pyrophosphatase in the RNA polymerase reactions was reduced to approximately 20% of that possible under optimal assay conditions. This inefficiency of the pyrophosphatase could be accounted for almost entirely by Mn^{2+} inhibition and by the pH shift from the optimum of 8·7 to 8·0 of the polymerase assays.[6,10] The activity of corn alkaline inorganic pyrophosphatase is dependent on added Mg^{2+} [6] thus depriving the RNA polymerase of some of its Mg^{2+} resulting in suboptimal polymerase activity. At concentrations above 2·5 mM Mg^{2+} RNA polymerase activity was inhibited.[4]

From the data we conclude that alkaline inorganic pyrophosphatase relieves exogenous pyrophosphate inhibition of chromatin-directed RNA synthesis *in vitro*. This supports Kornberg's proposal[2] that pyrophosphate hydrolysis promotes RNA biosynthesis. In the assays lacking exogenous pyrophosphate, however, the situation was less clear. We attribute the lack of resolution to the complex ion interactions in the coupled reactions chosen. It is possible that our results were influenced, to an unknown degree, by the varied sources of enzymes and DNA template. Experimental expediency and a desire to use higher plants as much as possible, guided us in the use of different organisms. *Zea mays* provided a concentrated source of alkaline inorganic pyrophosphatase, while the isolation and general characteristics of *Glycine max* chromatin and RNA polymerase are well established. Unfortunately the endogenous RNA polymerase activity of our soybean chromatin preparations was too low for the accuracy and replication we desired, so the assays were augmented with *E. coli* RNA polymerase. We hope that this work will be a stimulus for testing other reactions in an effort to verify Kornberg's proposal which is so often invoked.

EXPERIMENTAL

The extraction and partial purification of alkaline inorganic pyrophosphatase has been described.[6] The enzyme preparation used was that following calcium phosphate gel purification.

Chromatin was extracted according to Huang and Bonner[11] from the hypocotyls of 5-day-old etiolated soybeans (*Glycine max* L., var. Flambeau) grown in vermiculite at 28°. Following centrifugation through 2 M sucrose, the chromatin was washed twice with, and suspended in, 10 mM tris HCl (pH 8·0) containing

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5 mM Cleland's reagent. Aliquots of this suspension were used to assay chromatin-directed RNA synthesis as the incorporation of ^{3}H–UMP into trichlororoacetic acid (TCA) insoluble material. The reaction mixtures contained in μmoles: GTP, 0·1; CTP, 0·1; ATP, 0·1; UTP, 0·005; $MgCl_2$, 0·5; $MnCl_2$, 0·125; Cleland's reagent, 0·5; Tris HCl (pH 8·0), 10; and 7·5 μC ^{3}H-UTP (25·2 C/m-mole), 6 units *E. coli* RNA polymerase (Biopolymers Inc., Pinebrook, N.J.), and chromatin equivalent to 0·5 μg DNA in a total volume of 0·20 ml. Following incubation at 37° for 10 min the reactions were stopped by adding 4 ml ice-cold 10% (w/v) TCA containing 0·5 mM $Na_4P_2O_7$. After standing on ice for 20 min, the precipitates were transferred to membrane filters (Whatman, type GF/A) and washed with 25 ml cold 5% (w/v) TCA. The filters were dried and counted in a scintillation spectrometer using toluene containing 0·5% (w/v) 2,5-diphenyloxazole.

DNA was estimated by the diphenylamine reaction[12] on 0·8 N $HClO_4$ digests (90°, 20 min) of chromatin with deoxyadenosine as standard. Ribonuclease activity was measured according to Tuve and Anfinsen[13] at 37° for 30 min in 50 mM Tris HCl, 10 mM $MgCl_2$ at pH 8·7.

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Key Word Index—Zea mays; Gramineae; RNA polymerase; chromatin; alkaline pyrosphosphatase.