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Enzymatic Method for Continuous Monitoring of DNA Polymerase Activity[1]

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A simple and rapid method for the assay of DNA polymerase activity has been developed. The PP_i formation in the DNA polymerase reaction is continuously monitored by a coupled enzymatic method (P. Nyrén and A. Lundin, 1985, *Anal. Biochem.* **151**, 504–509) utilizing the enzymes ATP-sulfurylase and firefly luciferase. The method has been used for continuous monitoring of DNA synthesis *in vitro*, and the effect of an inhibitor, adriamycin, on the polymerase activity was studied. The assay is very sensitive and yields linear responses between 1.5 and 30 μg/ml of DNA polymerase (*Micrococcus luteus*) (2–40 pmol of PP_i generated per minute).

KEY WORDS: luminescence; bioassay; DNA polymerase; ATP-sulfurylase; DNA; nucleic acid chemistry.

DNA polymerase, the major enzyme in DNA synthesis, is found in all cells. The enzyme catalyzes the synthesis of DNA when all four deoxyribonucleoside 5′-triphosphates (dNTP)[2] are incubated with a primer DNA (with a free 3′-OH group) in the presence of Mg^{2+}. The reaction may be formulated as

$$(DNA)_n + dNTP \rightarrow (DNA)_{n+1} + PP_i$$

(n = number of residues).

The enzyme catalyzes not only the extension of DNA from the 3′-OH end group but also the exonucleolytic degradation of DNA from both 3′-hydroxyl end groups and 5′-end groups (1,2). DNA polymerase I from *Escherichia coli* consists of a single polypeptide chain with a molecular mass of 109 kDa (3). Klenow *et al.* (4) showed that this enzyme can be split by subtilisin into a 36-kDa fragment with all of the original 5′ → 3′ exonuclease activity and a 75-kDa fragment (the so-called Klenow fragment) with all of the polymerase and 3′ → 5′ exonuclease activities (4). Two assays (5) are normally used to measure polymerase activity: either a thymus DNA-primed method or a dAT-primed assay. Both assays measure the conversion of ^{14}C-, tritium-, or ^{32}P-labeled deoxyribonucleoside triphosphates into acid-insoluble products. Other methods of assay are (i) measurement of hypochromicity due to polymer formation, and (ii) measurement of PP_i formation. There is a need for a more rapid and more sensitive method for continuous monitoring of DNA synthesis, e.g., in the study of the initial events in DNA synthesis.

The aim of the present work was to utilize the sensitivity of the enzymatic method for PP_i analysis (6) for continuous monitoring of the DNA polymerase activity. The reactions occurring in the assay are

$$(DNA)_n + dNTP \xrightarrow{\text{DNA polymerase}} (DNA)_{n+1} + PP_i$$

(n = number of residues) [1]

$$PP_i \xrightarrow{\text{ATP-sulfurylase}}$$

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[2] Abbreviations used: dNTP, deoxyribonucleoside 5′-triphosphate; APS, adenosine 5′-phosphosulfate; BSA, bovine serum albumin; DTE, dithioerythriotol; PVP, polyvinylpyrrolidone M_r 360,000.

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$$PP_i + \text{APS} \rightarrow \text{ATP} + SO_4^{2-} \quad [2]$$

Luciferase

$$\text{ATP} + \text{luciferin} + O_2 \rightarrow \text{AMP} + PP_i + \text{oxyluciferin} + CO_2 + h\nu. \quad [3]$$

The K_m of the luciferase for ATP is 20 μM (7). The light emission is essentially time independent (decay rate <1% min) and proportional to the ATP concentration over the range 1×10^{-9}–0.5×10^{-6} M. Because the reaction rate in [3] within this concentration range is very low, only a negligible amount of PP_i is produced.

MATERIALS AND METHODS

Chemicals. Trizma base, DTE, magnesium acetate, BSA, APS, ATP-sulfurylase (ATP:sulfate adenylyltransferase; EC 2.7.7.4), DNA polymerase (deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase from *Micrococcus luteus;* EC 2.7.7.7), DNA (genomic type XV: activated from calf thymus), dNTP, and PVP 360,000 were purchased from Sigma (St. Louis, Mo.). PP_i was from Merck (Darmstadt, FRG). Purified luciferase (EC 1.13.12.7), D-luciferin, L-luciferin, and ATP standard were obtained from LKB Wallace (Turku, Finland). DNA polymerase I (large fragment, Klenow enzyme) was from Boehringer (Mannheim, FRG). Single-stranded bacteriophage M13mp7 (+)strand DNA and M13 single-stranded primer (17 bases) were from Pharmacia (Uppsala, Sweden).

Continuous monitoring of DNA-polymerase activity. The assay was performed using an LKB 1250 luminometer and 2210 potentiometric recorder (LKB Wallace). The luminometer was calibrated to give a response of 10 mV for the luminometer internal light standard. The luminescence output was calibrated by addition of a known amount of ATP or PP_i both before and after the assay. The reaction was carried out at room temperature. The standard assay volume was 1.0 ml and contained the following components: 0.1 M glycylglycine, pH 7.75, 2 mM EDTA, 10 mM magnesium acetate, 0.1% BSA, 1 mM DTE, 0.4 mg/ml PVP 360,000, 100 μg D-luciferin, 4 μg L-luciferin, 5 μM APS, 0.30 U ATP-sulfurylase, purified luciferase in an amount giving a response of 1 V for 0.1 μM ATP, 10 μg activated calf thymus DNA, or 0.5 or 5 μl of a mixture containing 25 mM Tris–Cl, pH 7.5, 2.5 mM $MgCl_2$, 0.056 μg/ml M13mp7 (+)strand DNA, 0.0075 A_{260} U/ml single-stranded primer (17 bases) which had been preincubated for 1 h at 55°C, 5 μM dNTP, and 7.7 μg DNA polymerase (*M. luteus*) or Klenow enzyme as indicated. A 10 times concentrated stock solution containing 0.1 M glycylglycine, pH 7.75, 0.1 M magnesium acetate, 1% BSA, 4 mg/ml PVP, 1 mg/ml D-luciferin, 40 μg/ml L-luciferin, and luciferase could be stored frozen at −20°C for at least a month.

RESULTS

All experiments with the DNA synthesis monitoring system was performed at pH 7.5. This pH is optimal for both the luciferase system (8) and the ATP-sulfurylase (9). The pH optima for the *M. luteus* DNA polymerase (10) and for the DNA polymerase I (*E. coli*) (11) are broad, from 7.6 to 8.2 and from 7.0 to 9.0, respectively. The first series of experiments was performed to find optimal concentrations of DNA template (activated calf thymus DNA) and dNTP for the continuous monitoring technique.

Figures 1 and 2 shows the influence of the concentrations of activated DNA and dNTP on the specific rate of DNA synthesis. The results are in accordance with Ref. (12). From these results (Figs. 1 and 2) 10 μg DNA/ml and 5 μM dNTP were chosen for use in the assay of DNA polymerase activity. It is important that dNTP solutions with low PP_i contamination are used. A concentration of enzyme corresponding to 1.5 to 30 μg/ml (*M. luteus*) resulted in a linear relationship between enzyme amount and bioluminescence (2–40 pmol of PP_i generated per minute).

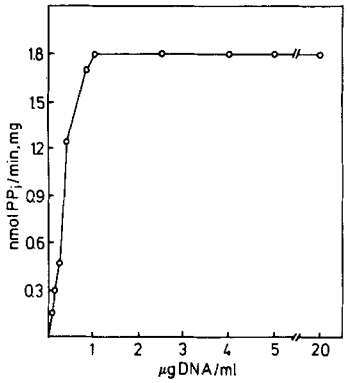

FIG. 1. Specific rate of DNA synthesis as a function of DNA concentration. Experimental conditions were as described under Materials and Methods. From the start, the reaction mixture contained 5 μM dNTP and 7.7 μg DNA polymerase in a total volume of 1.0 ml.

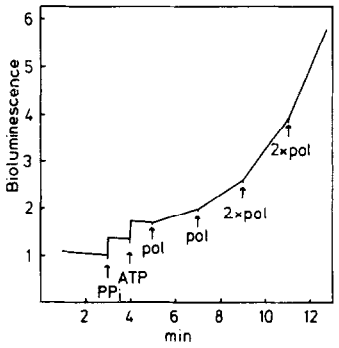

FIG. 3. Measurement of PP_i production during DNA synthesis. Experimental conditions were as described under Materials and Methods. From the start, the reaction mixture contained 5 μM dNTP and 10 μg activated DNA in a total volume of 1.0 ml. The following additions were made: 7.5 pmol PP_i, 7.5 pmol ATP, and 2 μg DNA polymerase.

The continuous monitoring of DNA synthesis at different DNA polymerase concentrations is illustrated in Fig. 3. As can be seen from this figure addition of DNA polymerase to a mixture with constant bioluminescence resulted in a linear increase of the light, and injection of 7.5 pmol ATP or PP_i into the assay mixture gave virtually the same light intensity. However, as PP_i is a product-inhibitor of the DNA polymerase reaction, it is more convenient to use ATP as an internal standard. The stability of the light output was due to the DTE present as shown before (6). Addition of 0.4 mg/ml PVP further increased the stability and gave an even lower decay rate, probably due to destabilization of the enzyme-product complex (not shown). The decay rate was below 1%/min up to 0.5 μM ATP. In subsequent experiments, the ATP concentration never exceeded 0.5 μM. The sensitivity of the method is clearly demonstrated in Fig. 4. In this experiment the

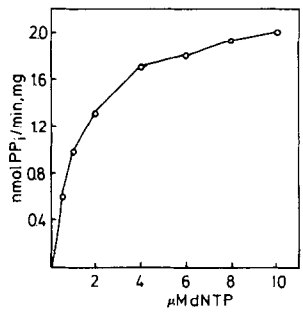

FIG. 2. Specific rate of DNA synthesis as a function of dNTP concentration. Experimental conditions were as described under Materials and Methods. From the start, the reaction mixture contained 10 μg activated DNA and 7.7 μg DNA polymerase in a total volume of 1.0 ml.

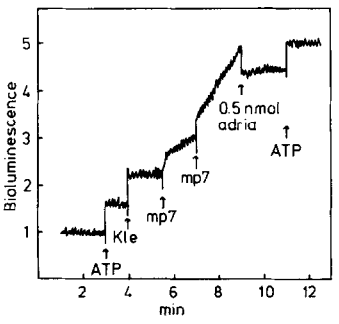

FIG. 4. Continuous monitoring of DNA polymerase activity with the single-stranded bacteriophage M13 as template. Experimental conditions were as described under Materials and Methods. The following additions were made: 0.5 nmol adriamycin, 74 ng DNA polymerase I (Klenow enzyme), 0.5 nmol ATP, and 0.5 μl mp7 mixture (25 mM Tris-Cl, pH 7.5, 2.5 mM $MgCl_2$, 0.056 μg/ml mp7 (+)strand, 0.0075 A_{260} U/ml single-stranded primer (17 bases) preincubated for 1 h at 55°C). From the start, the reaction mixture contained 5 nmol dNTP in a total volume of 1.0 ml.

single-stranded bacteriophage M13 was used as template. The DNA concentration in the reaction mixture was as low as 0.028 μg/ml, and a production of 0.3 pmol PP_i per minute was easily detected. The reaction was inhibited by 0.5 nmol adriamycin, a known DNA synthesis inhibitor (13). The inhibition of DNA synthesis by adriamycin was dependent on the DNA template concentration (Fig. 5) and could be reversed by addition of more DNA template. Note that the response of the luciferase system is slightly inhibited after addition of adriamycin. However, this inhibition is compensated for by addition of an internal ATP standard.

DISCUSSION

A new method for continuous monitoring of DNA synthesis is presented. The PP_i formation in the DNA polymerase reaction is continuously monitored by a coupled enzymatic method (6) utilizing the enzymes ATP-sulfurylase and firefly luciferase. By using this method, studies of the reaction kinetics of the DNA polymerase reaction under a variety of conditions (e.g., at different substrate concentrations and in the presence of different inhibitors) can be conveniently performed. This opens up new possibilities for obtaining a detailed picture of the events involved in DNA synthesis and the effects of different compounds on the polymerase activity. The method is very sensitive; a production of 0.3 pmol PP_i per minute can easily be detected (Fig. 4). Analytical interference due to sample compositions or reagent inactivation is easily detected and can generally be compensated for by internal calibration with a PP_i standard.

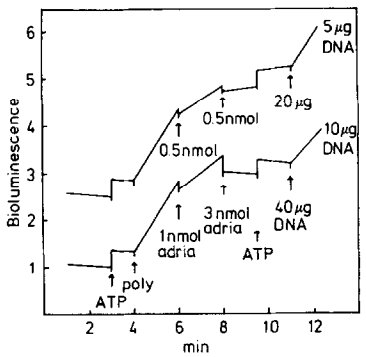

FIG. 5. Profile of DNA synthesis under different conditions. Experimental conditions were as described under Materials and Methods. From the start, the reaction mixture contained 5 nmol dNTP and 5 and 10 μg activated DNA, respectively (as indicated in a total volume of 1.0 ml). The following additions were made: 5.0 pmol ATP, 7.7 μg DNA polymerase, and adriamycin as indicated. Note that the response of the luciferase system is inhibited by 20% after addition of 4 nmol adriamycin. This inhibition is compensated for by addition of an internal ATP standard.

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