Apparent stimulation of calf thymus DNA polymerase alpha by ATP

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ABSTRACT

The mechanism by which millimolar concentrations of ATP stimulate the activity and increase the processivity of calf thymus DNA polymerase alpha has been investigated with poly(dA)/oligo(dT) as template/primer to eliminate possible effects due to primer synthesis. The effect of ATP on the rate of DNA synthesis with this template/primer was found to be dependent upon whether or not the ATP was neutralized and the species of buffer used in the reaction. The present studies suggest that ATP stimulation of calf thymus DNA polymerase can be attributed to changes in the pH of the reaction mixture, a shift in the magnesium ion optimum, or both. Furthermore, effects of ATP on the processivity of DNA polymerase alpha could be mimicked by lowering the pH of the reaction mixture.

INTRODUCTION

DNA-dependent hydrolysis of ATP plays an important role in the replication, repair and recombination of DNA in prokaryotes (1). In addition to its role in unwinding the DNA helix, ligating DNA fragments and the synthesis of primers, ATP has been shown to be required for the formation of an initiation complex between *Escherichia coli* DNA polymerase III holoenzyme and a template/primer (2,3). In the absence of DNA, ATP forms a tight complex with DNA polymerase III holoenzyme with a K_D of about 1 μ M. The apparent K_M for activation of DNA polymerase III holoenzyme with poly(dA)/oligo(dT) as template/primer is approximately 10 μ M (4).

In higher organisms ATP has also been found to stimulate DNA synthesis with DNA polymerase alpha, the species of DNA polymerase implicated in replication of the mammalian genome. Delineation of the role of ATP with DNA polymerase alpha is complicated by the fact that the enzyme is usually purified as a complex with a primase activity that utilizes ribonucleoside triphosphates as substrates for synthesis of oligoribonucleotide primers. However, under conditions where the synthesis of primers is apparently not a factor, relatively high concentrations of ATP, i.e., 3 to 5 mM, have been

found to markedly stimulate the rate of DNA synthesis (5,6,7,8). Studies with DNA polymerase alpha from calf thymus (5,6) and *Xenopus laevis* (9) have suggested that ATP increases the processivity of the polymerase by increasing the binding of the enzyme to the primer/template. However, the mechanism by which ATP affects the interaction of the polymerase with the template/primer is unclear.

In this communication we shall present data showing that the effects of millimolar concentrations of ATP on the activity and processivity of calf thymus DNA polymerase alpha, under assay conditions commonly used with this enzyme, can be accounted for by effects on the pH of the reaction mixture.

MATERIALS AND METHODS

Unlabeled deoxynucleoside triphosphates, poly(dA) ($S_{20,w}$ 6.7), (dT)₁₀, and the disodium salt of ATP were obtained from Pharmacia-PL Biochemicals. [³H]dTTP (50-80 Ci/mmol), [α -³²P]dTTP (650 Ci/mmol) and [γ -³²P]ATP (7000 Ci/mmol) were purchased from ICN Pharmaceuticals. Fetal calf thymus glands were from Bio-Resources Inc. Tris base and Tris hydrochloride were from Sigma Chemical Co. Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) was from Boehringer Mannheim Biochemicals. Hae III fragments of ϕ X174 RF DNA and T₄ polynucleotide kinase were from Bethesda Research Laboratories.

Poly(dA)/oligo(dT) (20:1) was prepared by annealing the template and primer in a weight ratio of 20:1 in 20 mM Hepes buffer, pH 7.4, 60 mM KCl at 80°C for 10 min, followed by slow cooling. DNA polymerase alpha was purified from fetal calf thymus essentially as previously described (10). On SDS-gel electrophoresis, the enzyme preparation contained multiple protein bands of 50 to 180 kD; major bands were at 135, 120 and 106 kD. This DNA polymerase alpha species has primase activity and would correspond to DNA polymerase alpha A as described by Holmes *et al.* (11). DNA-dependent and independent ATPase activity, assayed as previously described (10), was not detectable in the enzyme preparation. One unit of DNA polymerase activity is defined as the incorporation of 1 nmol dNMP/hr with activated calf thymus DNA as template, as previously described (10).

Stock solutions of Tris-HCl buffer (1.0 M) were prepared by titration of Tris base with Tris-HCl at 24°C. Stock solutions of Hepes buffer (1.0 M) were prepared by titration with KOH at 24°C. Diluted buffers (60 mM) were 0.1 to 0.2 pH units lower than the 1.0 M stock solutions, however, for convenience, assay pH conditions refer to the pH of the stock buffer. Un-

neutralized ATP (pH 4.0) was prepared by dissolving disodium ATP in double distilled H₂O. Neutralized ATP was prepared by titration of unneutralized ATP to pH 7.0 with NaOH.

DNA Polymerase Assay

The reaction mixture contained in a final volume of 0.125 ml: 0.1 A₂₆₀ unit poly(dA)/oligo(dT) (20:1); 60 mM Tris buffer, pH 7.5; 6 mM MgCl₂; 80 ug/ml bovine serum albumin; 4% glycerol; 40 uM dTTP (150 cpm/pmol) and 0.2 to 0.3 unit of DNA polymerase alpha. After 15 min at 37°C the reaction was stopped by the addition of 2 ml of cold 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected, washed and counted as previously described (12).

Processivity Determination

The processivity of DNA polymerase alpha under various assay conditions was determined by measuring the lengths of products formed in the presence of an excess of poly(dA)/oligo(dT) and total dTMP incorporation of less than 1 dTMP residue/molecule of primer (5,13). Reaction conditions were as given for the standard assay except that 0.42 A₂₆₀ unit poly(dA)/oligo(dT) (20:1) containing 300 pmol (dT)₁₀, [alpha-³²P]dTTP (2600 cpm/pmol) and 0.05 unit DNA polymerase alpha were used. Other conditions are given in the figure legend. Reactions were terminated by addition of EDTA to 50 mM and 10 ul aliquots were taken for determination of total incorporation. The remainder of the reaction mixtures were precipitated with ethanol for determination of product size. The precipitates were dissolved in TBE buffer (0.09 M Tris-borate, 4 mM EDTA, pH 8.3), made 60% in formamide, 2% in Ficoll, 0.02% in bromophenol blue and 0.02% in xylene cyanol FF. Samples were heated at 90°C for 5 min and chilled in ice. Electrophoresis was carried out on either 12% or 20% polyacrylamide, 7 M urea slab gels (16 x 14 x 0.075 cm) in TBE buffer according to Maniatis *et al.* (14). Hae III fragments of ϕ X174 RF DNA, 5'-labeled with [gamma-³²P]ATP and T₄ polynucleotide kinase according to the supplier's protocol, were run as size markers. Autoradiography was done using Kodak XAR film and a Dupont Cronex intensifying screen at -70°C.

RESULTS

Neutralized ATP Has Little Effect on DNA Synthesis

Figure 1 shows the effects of either neutralized or unneutralized ATP on the rate of DNA synthesis with calf thymus DNA polymerase alpha on a poly(dA)/oligo(dT) template/primer. In the presence of 60 mM Tris-HCl, pH 7.5, unneutralized ATP stimulates the rate of DNA synthesis about 8-fold at

Figure 1: Effect of neutralized and unneutralized ATP on the rate of DNA synthesis in the presence of Tris-HCl buffer. DNA polymerase activity was determined as described in Materials and Methods except for the addition of either neutralized (●) or unneutralized (o) ATP, as indicated.

a concentration of 3 mM, whereas with neutralized ATP (pH 7.0) the effect of ATP was considerably less; only a 3 fold stimulation was observed at 4 mM. However, if 60 mM Hepes buffer, pH 7.5, was substituted for Tris-HCl, pH 7.5, very little stimulation of DNA synthesis was seen with either neutralized or unneutralized ATP at concentrations ranging from 1 to 5 mM

Figure 2: Effect of neutralized and unneutralized ATP on the rate of DNA synthesis in the presence of Hepes buffer. The incubation conditions were as described in Figure 1 except that Hepes buffer, pH 7.5, was used instead of Tris-HCl buffer, pH 7.5. The symbols are as in Figure 1.

Figure 3: Effect of unneutralized ATP on the pH of Hepes and Tris buffers. The concentration of either Hepes (o) or Tris-HCl (●) buffer was 60 mM. The pH was measured at 24°C (panel A) or 37°C (panel B) with a Radiometer pH meter. The concentration of ATP was varied as indicated.

(Figure 2). These results suggest that the effect of unneutralized ATP on DNA synthesis with calf thymus DNA polymerase alpha might be a consequence of a change in the pH of the reaction mixture.

Unneutralized ATP Significantly Lowers the pH of Tris-HCl Solutions

The effects of increasing concentrations of unneutralized ATP on the pH of solutions of two different buffers, Tris with a pKa of 8.3 and Hepes with a pKa of 7.55 at 20°C, are shown in Figure 3. At 24°C (panel A), increasing concentrations of unneutralized ATP markedly lower the pH of the solution when Tris-HCl is the buffer (from 7.46 to 5.95), whereas with Hepes buffer, addition of unneutralized ATP results in a decrease of less than 0.2 pH unit. Since the dissociation constant of the buffer is affected by temperature, we also determined the effect of unneutralized ATP on the pH of the buffer solutions at 37°C, the temperature at which the polymerase reaction is carried out (panel B). At this temperature the effect of unneutralized ATP on the pH of the solution is less marked, however, it still decreased the pH of 60 mM Tris-HCl about 0.6 pH unit. In contrast, the pH of 60 mM Hepes buffer was lowered less than 0.1 pH unit at 37°C. These results suggest that Tris-HCl is not a very effective buffer at pH 7.5.

Influence of pH and ATP on the Magnesium Ion Optimum

The effects of varying Mg^{++} concentrations on the rate of DNA synthesis in Hepes buffer at either pH 7.0 or pH 7.5 are shown in Figure 4. It is apparent that the Mg^{++} optimum increases as the pH decreases. At pH 7.5, the optimal concentration of Mg^{++} is 0.5 mM and concentrations greater than

Figure 4: Effect of pH on the magnesium ion optimum. The assay conditions were as in Materials and Methods except that 60 mM Hepes buffer, pH 7.0 (o) or 7.5 (●), was used instead of Tris-HCl buffer, and the Mg^{++} concentration was varied as indicated.

1 mM are inhibitory, whereas at pH 7.0 the concentration of Mg^{++} required for maximal activity is broader and ranges from 2.5 to 6 mM. Figure 5 shows the effects of ATP on the Mg^{++} optimum in the presence of 60 mM Tris pH 7.5. In the absence of ATP, the concentration of Mg^{++} required for maximal activity was 0.5 to 1.0 mM. However, in the presence of 3 mM unneutralized ATP, the optimal Mg^{++} concentration was shifted to 4 mM. Thus, the effects

Figure 5: Effect of ATP on the magnesium ion optimum. The assay conditions were as described in Methods and Materials except for the presence (●) or absence (o) of 3 mM unneutralized ATP. The Mg^{++} concentration was varied as indicated.

Figure 6: Effect of pH on the processivity of DNA polymerase alpha. 12% (panel A) or 20% (panel B) polyacrylamide, 7 M urea gels of products synthesized in: Hepes buffer pH 7.0 (lane 1), Hepes buffer, pH 7.5 (lane 2), Tris-HCl buffer, pH 7.5 (lane 3) or Tris-HCl buffer, pH 7.5, plus 3 mM unneutralized ATP (lane 4) were run as described in Materials and Methods. In 12% gels bromophenol blue (BPB) comigrates with polymers of 12 nucleotides and xylene cyanol FF (XC) comigrates with polymers of 42 nucleotides. On 20% gels BPB comigrates with polymers of 8-10 nucleotides and XC with polymers of 28 nucleotides. The numbers indicate the size of [32 P]-labeled Hae III fragments of ϕ X174 RF DNA run in a parallel lane.

of ATP on the rate of DNA synthesis with poly dA/oligo dT could be attributed to a lowering of the pH and a shift of the optimal Mg^{++} concentration.

Effect of pH on the Processivity of DNA Polymerase Alpha

The effects of pH on the size of the products synthesized under conditions where only one polymerase-DNA binding event occurs, i.e., approx-

imately 0.2 pmol dTMP incorporated/pmol (dT)₁₀, are shown in Figure 6. In Tris-HCl buffer, pH 7.5, the products were less than 28 nucleotides in length, whereas in either Hepes buffer, pH 7.0, or in Tris-HCl, pH 7.5 with 3 mM unneutralized ATP, much larger products were seen. The product size distribution in Hepes buffer, pH 7.5, was intermediate between that seen in Hepes, pH 7.0, and Tris-HCl, pH 7.5. These results suggest that, with poly(dA)/oligo(dT) as template/primer, DNA polymerase alpha is more processive at pH 7.0 than at pH 7.5, and that the increased processivity seen in the presence of unneutralized ATP when Tris-HCl, pH 7.5, is the buffer, is the result of lowering the pH.

DISCUSSION

Recent studies have shown that DNA polymerase alpha species from calf thymus and mouse Erlich ascites cells are stimulated by millimolar concentrations of ATP (5,6,7,8). In these studies, as in much published work with DNA polymerase alpha, the polymerase activity was determined in a reaction mixture in which 20-60 mM Tris-HCl buffer was used to maintain the pH at 7.4 to 7.6. The concentration of ATP required for effective stimulation of DNA synthesis was in the millimolar range, a concentration considerably higher than that required for replication of DNA by *E. coli* DNA polymerase III (4).

It appears unlikely that the stimulation of DNA synthesis by high concentrations of ATP is mediated through the synthesis of RNA primers by the primase activity associated with DNA polymerase alpha in the case of primed homopolymer templates such as poly(dA)/oligo(dT) (3,4). In studies with MVM viral DNA (7,8), the conversion of single-stranded DNA to double-stranded DNA has been demonstrated to be self-primed but the possibility that the stimulation of DNA polymerase alpha activity by ATP might be mediated through the synthesis of RNA primers has not been eliminated. However, the optimal rNTP concentration for the primase activity of DNA polymerase alpha has been shown to be approximately 0.5 mM, a concentration 8- to 10-fold lower than the optimal concentration for ATP stimulation of DNA polymerase activity.

The present studies show that ATP stimulation of calf thymus DNA polymerase alpha activity with poly(dA)/oligo(dT) as template/primer may be due to a dual effect on the pH optimum and the Mg⁺⁺ requirement. An apparent stimulation can only be demonstrated if ATP is not neutralized and

the buffer used is ineffective in maintaining the pH of the reaction mixture, as in the case of Tris-HCl, pH 7.5 (pKa 8.3). With neutralized ATP, considerably less stimulation is observed. When Hepes buffer, pH 7.5 (pKa 7.55) is substituted for Tris-HCl, pH 7.5, little or no stimulation is observed with either neutralized or unneutralized ATP.

The present studies further demonstrate that the Mg^{++} concentration that is optimal for DNA polymerase alpha activity on primed homopolymer templates varies as a function of pH; at pH 7.0, the Mg^{++} optimum is 2.5-4.0 mM, whereas at pH 7.5 it is approximately 0.5 mM. As a consequence, the addition of an agent that lowers the pH of a reaction mixture in which the Mg^{++} concentration is 5 to 10 mM would result in an apparent stimulation of DNA polymerase activity. It is noteworthy that Hesslewood *et al.* (15) and McKune and Holmes (16) have shown that several species of DNA polymerase alpha from calf thymus exhibit optimal activity with poly(dA)/oligo(dT) template/primers at pH 6.5 to 6.8 and Mg^{++} concentrations of 6 to 8 mM at this pH.

The effect of unneutralized ATP on the processivity of DNA polymerase alpha with poly(dA)/oligo(dT) as template/primer can be mimicked by lowering the pH of the reaction mixture. Furthermore, when single-stranded M13mp19 DNA, annealed to a 17-base primer, was used as template/primer, significantly larger products were synthesized at pH 6.5 than at pH 7.5 (unpublished observation), suggesting that the increased processivity of DNA polymerase alpha seen in the presence of ATP in previously published studies (5,6,9) was mediated through pH effects. It should be emphasized that the present results were obtained with purified DNA polymerase alpha and, as a consequence, accessory proteins or subunits which require ATP for activation or which utilize hydrolysis of ATP for energy-requiring processes might not be present. Furthermore, it is not known whether the ATP used in previously published studies was neutralized.

Although the role of ATP in DNA replication in eukaryotes is still not fully understood, it is possible that *in vitro* effects of ATP which are manifested at high ATP concentrations are due, at least in part, to changes in the pH of the reaction mixture as well as changes in the Mg^{++} optimum that result from the addition of unneutralized ATP. The mechanism by which the pH of the reaction mixture affects the interaction of the polymerase with the template/primer is unclear, since pH might affect the conformation of the enzyme, the secondary structure of the template/primer, or both.

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