THERMAL SENSITIVITY OF EUKARYOTIC DNA POLYMERASE-α AND PROTECTION BY ITS TEMPLATES

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1. Introduction

Eukaryotic cells have been shown to contain at least three types of DNA polymerase designated as α-, β-, and γ-enzymes. Of these, in a variety of experimental systems, DNA polymerase-α was found to be the major enzyme involved in the replication process [1–7]. We have detailed the catalytic properties of DNA polymerase-α from regenerating rat liver [8–10] and more recently we have reported our data concerning the structural characterization of the purified enzyme [11]. Since the α-enzyme can use different kinds of DNA templates, we have studied its heat sensitivity as a means to further evaluate a differential binding of the enzyme to these templates. Due to the numerous changes which can occur during thermal denaturation of a protein and the complications arising from multiple equilibria of amino acid residues or individual subunits involved (reviewed [12]), we have not yet investigated the denaturation aspect in terms of physical properties and molecular conformation. However, we would emphasize that the thermal treatment of the α-enzyme was used here as a probe to provide new insight regarding its catalytic properties, rather than aiming at an understanding of its denaturation process itself.

Here we report the effects of thermal inactivation on the ability of the enzyme to use a DNA or RNA primer for initiation of DNA synthesis and the protective effect of the DNA template on the rate of polymerase inactivation.

2. Material and methods

2.1. *Materials*

All materials were exactly as in [9,11]. The experiments reported here were all performed with purified DNA polymerase-α, possessing spec. act. $3–5 \times 10^4$ units . mg^{-1} . h^{-1} [11].

2.2. *DNA polymerase assays*

Unless otherwise stated, assays using activated calf thymus DNA (350 μg/ml) were at 37°C in 50 mM Tris–HCl (pH 8.1), 8 mM $MgCl_2$, 5 mM KCl, 5 mM 2-mercaptoethanol, 15% glycerol, 450 μg/ml bovine serum albumin, 100 μM each of dATP, dGTP, dCTP, and d[^{3}H]TTP (100–400 cpm/pmol), in 50 μl total vol. Assays using poly(dC) · $(dG)_{12-18}$ (50–100 μM) were in 50 mM Tris–HCl (pH 8.1), 1.5 mM $MgCl_2$, 5 mM KCl, 5 mM 2-mercaptoethanol, 15% glycerol, 450 μg/ml bovine serum albumin, and 75 μM d[^{3}H]GTP (100–300 cpm/pmol). In the RNA-primed poly(dT) · $(A)_{10-20}$ (50–100 μM) assay, the reaction mixture contained 50 mM Tris–HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 15% glycerol, 75 μM, d[^{3}H]ATP (100–300 cpm/pmol). Incorporation of labeled nucleotide into acid-insoluble material was measured as in [8]. One unit of DNA polymerase-α activity was defined as the amount of DNA polymerase required to convert 1 nmol total nucleotide into acid-insoluble product in 1 h at 37°C, using activated calf thymus DNA as template. Reactions were for 15 min at 37°C.

2.3. *Heat inactivations*

In all the experiments, the α-polymerase was heat inactivated in a buffer containing 50 mM Tris–HCl (pH 7.6), 10 mM KCl, 5 mM 2-mercaptoethanol, 0.2 mM EDTA, 45% glycerol and 1 mg/ml bovine serum albumin. Samples (5 μl or 10 μl) were withdrawn at the times indicated in the legends to the figures, and were assayed with the appropriate DNA template as indicated above. Control samples were

taken from heat inactivation mixtures at zero time to give the initial activity of the enzyme. All the assays were done in duplicate or triplicate.

3. Results

3.1. *Kinetics of thermal inactivation of DNA polymerase-α*

As observed in [13,14], DNA polymerase-α was found to be heat-sensitive. The rate of heat inactivation of the α-enzyme was studied at 50°C, and the remaining catalytic activity was determined with activated DNA as template. As shown in fig.1, an initial inactivation corresponding to ~50% loss of enzyme activity occurred in the first 5 min and was then followed by an inactivation at a slower rate. The biphasic nature of the curve was also observed at the other preincubation temperatures tested (37°C and 45°C, not shown), but as expected, the time required for 50% loss of enzyme activity was related to the preincubation temperature. This result indicates that a major conformational change concerning its catalytic activity occurs in the molecular structure of the enzyme during the first step of thermal denaturation. No reversal of the inactivation was observed after the partially inactivated enzyme (half-inactivated at 50°C) had been stored at 4°C for as long as 6 h following the heating procedure (not shown).

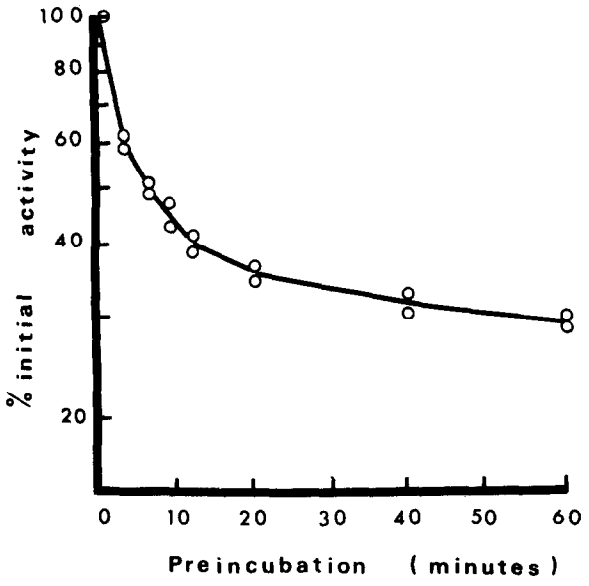

Fig.1. Heat inactivation of DNA polymerase-α. DNA polymerase-α (6.8 units) was incubated at 50°C in 90 μl 50 mM Tris–HCl (pH 7.6), 10 mM KCl, 5 mM 2-mercaptoethanol, 0.2 mM EDTA, 45% glycerol and 1 mg/ml bovine serum albumin. Samples (5 μl) were withdrawn at the indicated times and immediately assayed using actived calf thymus DNA, as in section 2. 100% initial activity was 79 pmol incorporated.

3.2. *Ability of DNA polymerase-α to use different kinds of template-primers after the thermal treatment*

Thermal inactivation kinetics experiments were performed and the residual activity was tested with different templates, i.e., activated calf thymus DNA, poly(dC) · $(dG)_{12-18}$ and poly(dT) · $(A)_{12-18}$. Figure 2 shows that the time course of denaturation, as detected by the residual α-polymerase activity, was nearly identical. Thus the ability of α-polymerase to use DNA templates of different base sequences in the

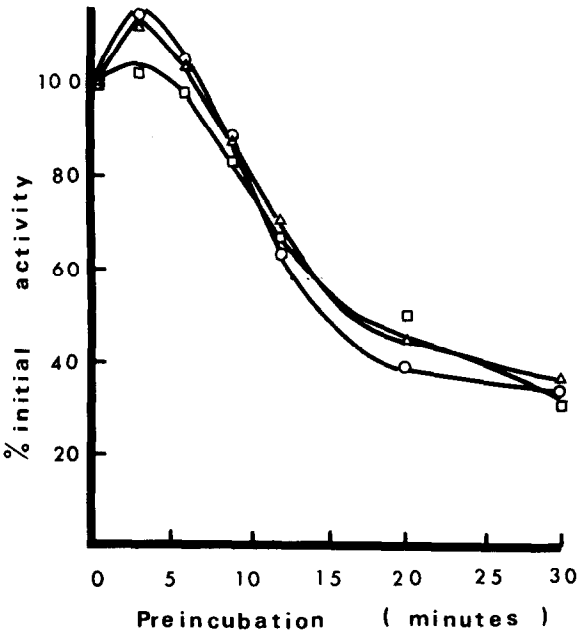

Fig.2. Thermal inactivation of DNA polymerase-α activity for various template-primers. DNA polymerase-α (52 units in 275 μl) was incubated at 45°C as in fig.1. Aliquots (5 μl) were withdrawn at the indicated times and added to 5 μl of the ice-cold buffer used for the heat treatment. When all samples were collected, the residual DNA polymerase activities were tested as in section 2 using activated calf thymus DNA (□——□, 100% activity was 188 pmol), or poly(dT) · $(A)_{10-20}$ (△——△, 100% activity was 66 pmol), or poly(dC) · $(dG)_{12-18}$ (○——○, 100% activity was 125 pmol).

presence either of a DNA or RNA 3′-hydroxyl primer to initiate the synthesis, was equally sensitive to the heat treatment. The slight activation of the enzyme detected during the first minutes of heat treatment was observed in some experiments, but not reproducibly. It could be due to a dissociation of inactive aggregated molecules of the stored purified enzyme during the first phase of heat treatment. Since the experiments were carried out in parallel, the kinetic curves obtained could be analyzed together.

3.3. *Protection of the α-polymerase by its DNA templates*

It was of interest to investigate whether different template-primers protect the enzyme against heat inactivation, since this would be a first indication of its template-primer affinity. As shown in fig.3, the presence of activated DNA or poly(dT) · $(A)_{10-20}$, at their optimal concentration for the catalytic activity during the heat treatment, afforded a great protection of the α-enzyme against thermal denaturation. Since this protection occurs in the absence of dNTPs and $MgCl_2$, it can be concluded that the binding of the α-enzyme to the template-primer occurs in the absence of the other substrates necessary for the catalytic activity itself. This finding was consistent with the fact that the α-polymerase could be purified by DNA-cellulose, as shown in [9–11].

The slight increase in catalytic activity of the enzyme preincubated in the presence of the DNA

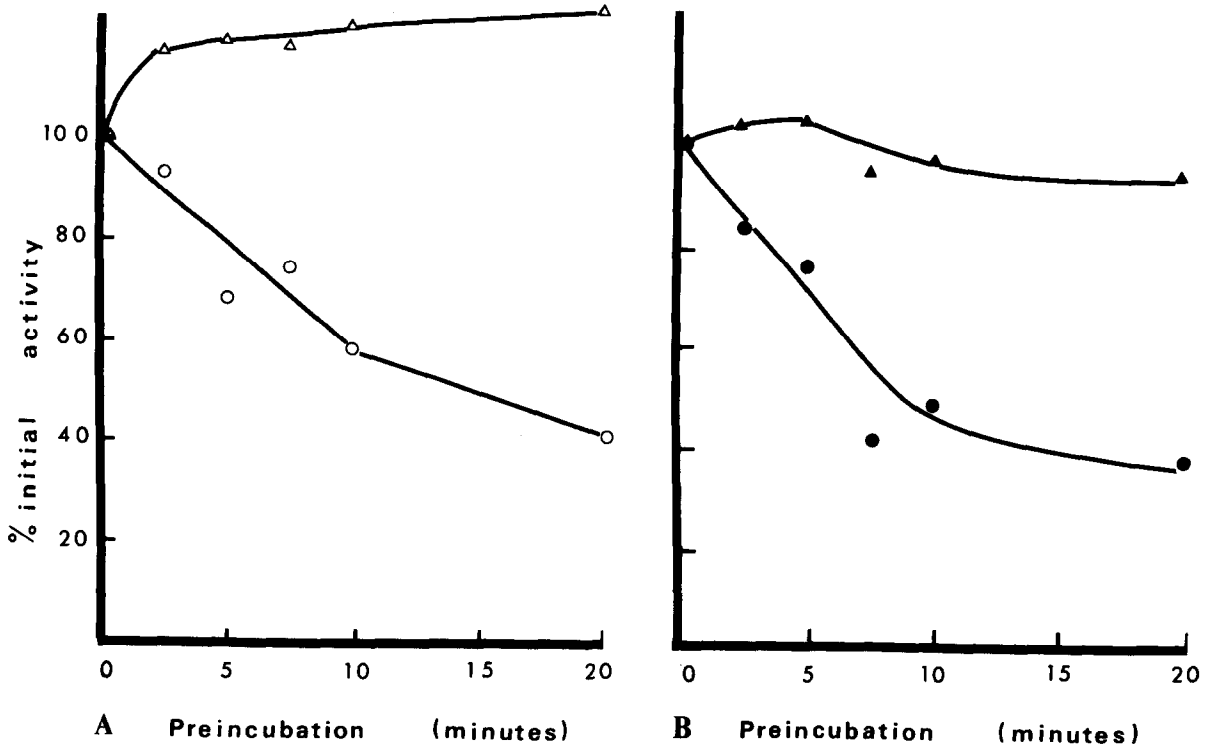

Fig.3. Protection of DNA polymerase-α-against heat inactivation by activated calf thymus DNA or poly(dT) · $(A)_{10-20}$. (A) A solution containing 35 units DNA polymerase-α in 130 μl of the same buffer as in fig.1 was separated in two equal fractions. Preincubation was then carried out at 37°C with (△——△) or without (○——○) added activated calf thymus DNA (375 μg/ml). Samples (5 μl) were withdrawn at the indicated times and assayed using activated calf thymus DNA, as in section 2. The activated DNA level in the polymerase assay was adjusted when relevant. 100% initial activity corresponded to 244 and 251 pmol incorporated in samples taken at zero time from inactivation mixtures without and with added activated DNA, respectively. (B) 35 units of DNA polymerase-α were treated as above. Preincubation was carried out at 37°C with (▲——▲) or without (●——●) added poly(dT) · $(A)_{10-20}$ (0.1 mM). Samples (5 μl) were withdrawn at the indicated times and assayed using poly(dT) · $(A)_{10-20}$ as in section 2. The poly(dT) · $(A)_{10-20}$ level in the polymerase assay was adjusted when relevant. 100% initial activity corresponded to 68 and 87 pmol incorporated in samples taken at zero time from inactivation mixtures without and with added poly(dT) · $(A)_{10-20}$, respectively.

template was reproducibly obtained during the heat treatment at 37°C. It can be explained by a pre-binding of the enzyme to the DNA template resulting in a more reactive conformation before the polymerization occurs. It was also interesting to note that, in presence of the DNA template, the binding site of DNA polymerase-α for the deoxynucleoside triphosphates was not affected by the heat treatment, since no loss of activity occurred.

4. Discussion

The time courses of thermal inactivation of the α-polymerase, observed when the remaining catalytic activity was used to follow the inactivation process, can be compared with the result obtained with the calf thymus α-enzymes C and D concerning their *N*-ethylmaleimide sensitivity [13,15]. In the latter, courses of inactivation were also found to be biphasic, with a rapid initial phase of inactivation, and a protective effect of the DNA templates was also observed. Although the two experimental modes of inactivation used (*N*-ethylmaleimide sensitivity or heat sensitivity) are clearly different, the results suggest at least two states of sensitivity of the enzyme relative to the inactivating treatment. Since the first step of denaturation rapidly reduces the α-polymerase activity but does not eliminate it (see fig.1), the nature of the transition state which occurs during the second part of the thermal denaturation kinetics remains a question.

The kinetics of thermal inactivation of the α-enzyme was nearly identical when different kinds of template-primers were used to test the residual catalytic activity (fig.2). This result may be interpreted to suggest that:

(i) The binding site to DNA templates was not base-specific;
(ii) The binding site of DNA polymerase-α to a DNA or RNA primer was the same one affected by the heat treatment; or
(iii) It was the deoxynucleoside triphosphate binding center which was inactivated.

Another possibility remains that the catalytic center of DNA polymerase-α involves different subsites for different template-primers which were inactivated at the same rate. Clearly, however, further analysis of the effects reported here will be necessary to investigate the conformational changes occurring within the enzyme during heat treatment.

The protection against heat inactivation by the DNA template indicates that DNA polymerase-α can bind to DNA in the absence of other substrates necessary for catalytic activity. This suggests that the binding of the enzyme to the template was the heat-sensitive step since it protects the catalytic center of the α-enzyme against heat inactivation. Moreover, this binding in the absence of dNTPs did not inhibit the activity of the enzyme when the polymerizing activity was further allowed to proceed.

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