FUNCTIONAL ASPECTS OF ZINC IN YEAST RNA-POLYMERASE B

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1. Introduction

It can be taken as fact that most RNA polymerases isolated from many biological species including procaryotic sources [1,2] yeast, algae, plants or mammals [3–9] are zinc proteins. Stoichiometries between one and seven moles of zinc per mole of enzyme were reported for these metal proteins. Unfortunately little is known about the functional side of the enzyme bound zinc in the catalysis of RNA formation.

For example, yeast RNA-polymerase B was found to contain one tightly bound zinc [6]. As in the case of *Escherichia coli* RNA-polymerase [1] treatment with 1,10-phenanthroline resulted in the loss of the overall enzymic activity. This rather limited information on the biochemical action of the protein bound zinc encouraged us to examine the inhibitory action of 1,10-phenanthroline in more detail. The DNA-nucleotide binding, the ternary complex with the yeast enzyme and the pyrophosphate exchange were studied in the presence of different concentrations of this chelator.

The action of 1,10-phenanthroline was different on DNA binding, formation of a ternary DNA-nucleotide–enzyme complex and on the overall reaction and its reversal the pyrophosphate exchange. The involvement of the enzyme bound zinc in the transfer of the entering nucleotide to the 3′-terminus of the growing RNA chain was suggested. This conclusions extends our knowledge on the originally proposed reactivity of zinc in the initiation of RNA synthesis [1] to the level of elongation and even translocation.

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2. Material and methods

2.1. *Material*

If not otherwise indicated chemicals and buffers used were the same as given in [6]. Fractions of DNAase-treated Ehrlich-ascites DNA labelled with [^{3}H]thymidine were a gift from U. Weidle from this institute.

2.2. *DNA binding assay*

The methods used were: a nitrocellulose binding and a gel filtration assay. In the filter binding assay a [^{3}H]DNA fraction renatured by heating and slowly cooling was chosen which was poorly retained by the filter (mol. wt approx. 70 000). RNA polymerase and DNA were mixed at 25°C applied to the buffer equilibrated filter and filtered under suction 2 min later. The filters were rapidly washed three times with standard buffer (see legend to fig.1) dried and counted in a toluene-based scintillator.

For the gel filtration a [^{3}H]DNA fraction not excluded by the gel (corresponding to less than 100 nucleotides) was mixed with RNA polymerase and glycerol and then applied to a column of Sephadex G-150 (1 × 50 cm) previously equilibrated with standard buffer. Total and acid insoluble radioactivity comigrating with the enzyme in the void volume were separately determined in each fraction.

2.3. *Complex formation with DNA and nucleotides*

In general filter binding and gel filtration methods

were used as depicted above. Preincubation of enzyme, unlabelled calf-thymus DNA and the respective nucleotide (1 μCi) was carried out in the presence of an at least ten-fold excess of Mn^{2+} over nucleotide. For the better separation of free and enzyme-bound nucleotides gel filtration was performed on Sephadex G-25.

2.4. *Pyrophosphate exchange*

RNA-polymerase was incubated in pyrophosphate exchange buffer (see legend to fig.3) in the presence of 1 mM each GTP, CTP, UTP, and 4.5 μCi $^{32}PP_i$ (final vol. 100 μl). The reaction was stopped by cooling and subsequent addition of excess EDTA together with unlabelled PP_i, followed by norite a. After shaking for 10 min the mixture was transferred into a column. Labelled PP_i was washed out and the labelled nucleotides were eluted with 1.5% NH_3 solution and counted in a Triton–toluene scintillator.

Determination of RNA-polymerase activity was carried out in this system with ATP, GTP, CTP, and UTP each 1 mM and 1 μCi [^{3}H]UTP per test using the procedure given in [6].

3. Results and discussion

3.1. *Complex formation with DNA and nucleotides*

The action of 1,10-phenanthroline on the formation of a complex between RNA-polymerase and double-stranded DNA is shown in fig.1. Using the gel filtration method and denatured DNA similar results were obtained: even in the presence of 30 mM inhibitor 70% of the labelled DNA paralleled the enzyme migration. Though still binding 90% of the DNA at 10 mM 1,10-phenanthroline the enzyme had lost 75% of its zinc content during the gel filtration with a concomitant loss in specific activity.

Formation of a ternary complex of enzyme, DNA and nucleotides was found to be inhibited by similar concentrations of 1,10-phenanthroline as above (fig.2). The two methods used showed a significant binding only with ATP and GTP. 10 mM inhibitor resulted in less than 50% inhibition of complex formation. Without added DNA, a significant binding could not be found for anyone of the four common ribonucleotides.

These results clearly show that blocking or removal of the enzyme-bound zinc neither decreases the binding of the template DNA nor the subsequent binding of an initial nucleotide to a greater extent.

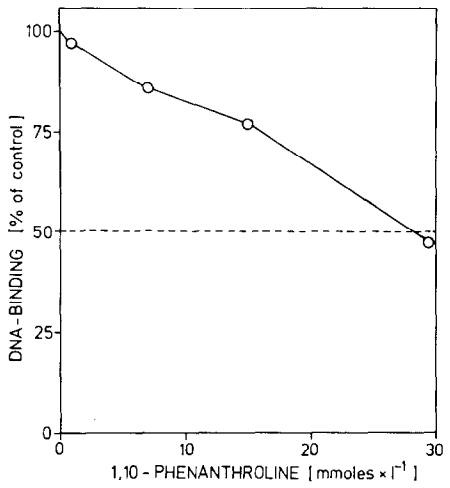


Fig.1. Inhibition of complex formation with native DNA by 1,10-phenanthroline. 5.5 μg polymerase (5 μl), 50 μl standard buffer (50 mM Tris–HCl, pH 7.8, at 25°C, 7 mM 2-mercaptoethanol, 30% (v/v) glycerol, rendered zinc-free by passage through a column of Chelex-100) were incubated with the indicated concentrations of inhibitor for 15 min. Then ^{3}H-labelled DNA was added (corresponding to 800 cpm) and the mixture applied to a buffer-equilibrated nitrocellulose filter. Two minutes after unbound DNA was removed by filtration and washing three times with standard buffer, radioactivity retained by the filter was determined. The control corresponded to 800 cpm, the blank (without polymerase) to 200 cpm.

3.2. *Pyrophosphate exchange*

Inhibition studies on the exchange of inorganic pyrophosphate were carried out to examine whether or not 1,10-phenanthroline is capable to displace the growing RNA chain and/or suppresses the binding of the starting nucleotide. Apart from the intriguing inhibitor dependent DNA binding studies given above a remarkable difference in the concentration of 1,10 phenanthroline required to yield 50% inhibition of two partial reactions is seen. In contrast to the inhibition of the overall reaction catalysed by the RNA-polymerase B the question arises whether the direct reversal of the last step might be inhibited in the same manner as observed for the whole reaction sequence. This result could be interpreted in that the enzyme-

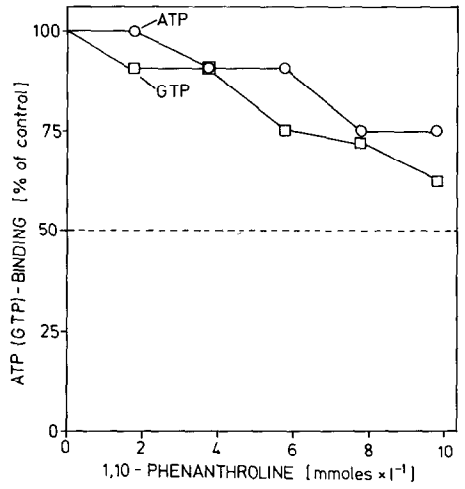
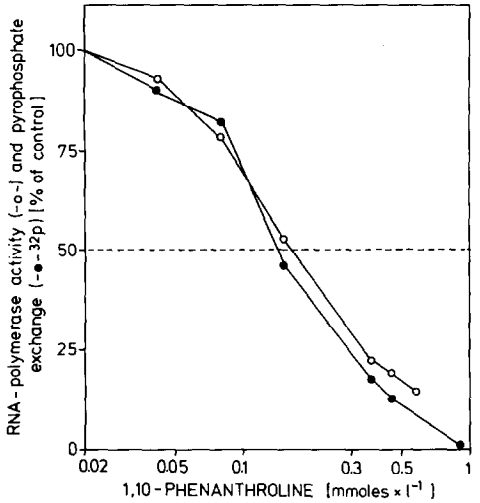


Fig.2. Inhibition of formation of a ternary complex DNA-polymerase nucleotide by 1,10-phenanthroline. Polymerase (11.3 μg), DNA (30 μg), $MnSO_4$ (1.4 nmol), ^{3}H-labelled nucleotides (20–60 pmol, 0.6–1 μCi) were incubated with the indicated concentrations of 1,10-phenanthroline for 5 min at 25°C and applied to a nitrocellulose filter. Two minutes after unbound nucleotides were removed by filtration under suction and washing with buffer and inhibitor. Filter-retained radioactivity corresponded to 70 ± 6 (80 ± 6) cpm for the ATP (GTP) control and 20 cpm for the blank.

bound zinc participates in the transfer of the newly entering nucleotide to the growing RNA chain by activating the 3′-OH of the ribose at its end.

Indeed, the pyrophosphate exchange (or pyro-



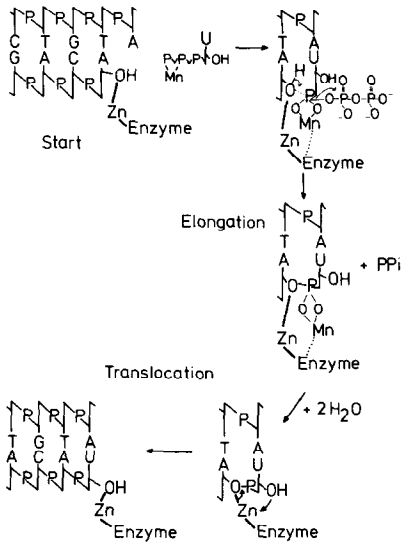


Fig.4. Proposed reaction mechanism of Zn–RNA–polymerase B.

phosphorolysis of very short RNA chains) was found to be inhibited to 50% by 1,10-phenanthroline at 0.14 mM with the overall reaction showing the same inhibition (50% at 0.17 mM) measured under identical conditions (fig.3). No inhibition was seen when the non-zinc binding analogous phenanthroline, 1,7-phenanthroline was used.

The relatively unspecific inhibition of template and nucleotide binding by 1,10-phenanthroline, and in contrast the very specific and identical inhibition

Fig.3. Inhibition of PP_i exchange and RNA synthesis by 1.10-phenanthroline. RNA polymerase (11 μg), $Na_4{}^{32}P_2O_7$ (4.5 μCi), and DNA (18 μg) were incubated with 1 mM each UTP, GTP, CTP, 0.73 mM $MnSO_4$, 45 mM Tris–HCl, pH 7.8, at 25°C, 45 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 1 mM dithiothreitol and inhibitor in final vol. 110 μl for 20 min at 37°C. The reaction was stopped by addition of 0.1 M EDTA, containing 50 mM unlabelled $Na_4P_2O_7$, pH 7.8. Labelled nucleotides were adsorbed to charcoal by shaking for 10 min. $^{32}PP_i$ was removed by washing the charcoal with unlabelled PP_i (10 mM) in 50 mM KH_2PO_4 solution (pH 6.0) in a small column. Thereafter, the nucleotides were eluted with 1.5% NH_3 solution and counted in a Triton–toluene scintillator. RNA synthesis was measured in the presence of additional ATP (1 mM) and 0.5 μCi of [^{3}H]UTP instead of labelled PP_i. Acid insoluble radioactivity after the incubation was determined by adsorption to a glass-fiber filter as in [6].

of forward and backward reaction in the last step of the formation of RNA by the RNA-polymerase B from baker's yeast support the proposal [10,11] that the function of the enzyme bound zinc in both RNA- and DNA-polymerases is the activation of the hydroxyl group of the sugar moiety to facilitate a nucleophilic attack of the primer or growing chain towards the α-phosphate group of the entering nucleotide (fig.4).

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