YEAST RNA-POLYMERASE B: A ZINC PROTEIN

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1. Introduction

Zinc deficiency is known to produce retarted growth and development in higher organisms and procaryotes [1–6]. Thus, it was not surprising that zinc has been found to be tightly bound in bacterial (*E. coli*) and viral polynucleotide synthesizing enzymes [7–14]. As most studies deal with enzymes from procaryotic sources, it was of interest to examine the role of zinc in the transcription machinery using an eucaryotic system. We chose the RNA-polymerase B from *Saccharomyces cerevisiae* where only one RNA-polymerase B activity has been found [15–17].

A large scale isolation procedure for RNA-polymerase B from baker's yeast is described, which permits to the minimization of metal contamination. This method yields, in a relatively short time, milligram amounts of homogeneous enzyme. The zinc content, its correlation with the specific activity, and inhibition experiments led us to conclude, that the DNA-dependent RNA-polymerase from baker's yeast is a Zn-protein with a 1:1 stoichiometry.

2. Materials and methods

2.1. *Material*

All chemicals used were of analytical grade. Contamination with metal ions was minimized by employing only polypropylene, -acryl, and -ethylene ware. Deionized water was additionally distilled over quartz (conductivity < 2 μmho, Zn < 1 nM).

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Standard buffer contained 50 mM Tris, 0.1 mM EDTA, 7 mM β-mercaptoethanol and 15% glycerol (v/v), pH 7.8 at 20°C. Homogenization buffer additionally contained 5 mM $MgCl_2$ and 50 mg/l phenylmethylsulfonylfluoride. Dialysis buffer was standard buffer made up with 60% glycerol (v/v). All buffers used (except for the first purification steps) were metal freed by chromatography (without added EDTA and mercaptoethanol) on chelating resin from 100–300 nM down to less than 10 nM. DNA-agarose was prepared according to [18]. Baker's yeast was from Lindenmayer & Co., Heidelberg or Sinner KG, Karlsruhe. No changes in enzyme content due to different physiological state of the cells could be seen.

2.2. *Analytical procedures*

Protein was determined according to [19]. Polyacrylamide gel electrophoresis under non-denaturing conditions was carried out using the gel system 1a [20] and a 6% separation gel. SDS polyacrylamide gel electrophoresis was performed in 7.5% gel [21]. Zinc was assayed by atomic absorption spectrometry (Perkin Elmer model 300S, graphite cell).

RNA-polymerase assay. The standard assay mixture, pH 7.8 contained in 125 μl: 25 μl enzyme (0.1–10 μg); 0.5 mM of each ATP, CTP, and GTP; 0.1 mM [^{3}H] UTP (0.5 μCi, 40 Ci/mole); 25 μg heat-denatured DNA; 50 mM Tris; 50 mM $(NH_4)_2SO_4$; 0.1 mM EDTA; 1 mM dithiothreitol; 1.6 mM $MnSO_4$. After incubation at 37°C for 10 min, the assay mixture was cooled in an ice-bath and 50 μl of RNA-PP_i (2 mg/ml RNA in saturated sodium pyrophosphate solution at pH 7.8) were added. A 100 μl aliquot was pipetted onto a glass fiber, fixed to a stainless steel frame. The filter loaded frame was placed at 23°C for 5 min each into

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6 successive baths, containing 5% sodium pyrophosphate solution (saturated) plus 4% (bath 1) or 0.8% (baths 2–4) trichloroacetic acid, and methanol (baths 5,6). The filters were dried at 90°C for 15 min and the radioactivity determined using a toluene-based scintillator. One unit of activity corresponds to the incorporation of 1 nmol [^{3}H]UTP into acid-insoluble product in 10 min under the conditions given above.

3. Results and discussion

3.1. *Large scale preparation of homogeneous RNA-polymerase B*

The methods given in [22–24] were modified as follows: 1500 g of frozen yeast cells were thawed in 1.5 litre of homogenization buffer. The suspension was chilled to −10°C. Cell cracking was achieved by two passes under chilling through the pressure chamber of a Manton-Gaulin homogenizer maintained at 530 atm. Cell debris and whole cells were removed by centrifugation (10 min, 2500 rev/min, Mistral 6L). The supernatant was further centrifuged at 17 000 *g* for 2 h (Beckman J 21). The lipid layer was separated from the supernatant by filtration through a cheesecloth and then solid $(NH_4)_2SO_4$ was slowly added to yield a concentration of 180 mM, the pH was 8.0/4°C using NH_3-solution.

In the first DEAE-chromatography the supernatant was loaded on a column, 8 × 40 cm, equilibrated with standard buffer + 180 mM $(NH_4)_2SO_4$. The column was extensively washed with the same buffer at a flow rate of 300 ml/h until no more contaminating protein including RNA-polymerase A was eluted as shown by the absorbance at 253 nm. The adsorbed proteins were eluted with 300 mM $(NH_4)_2SO_4$ in standard buffer. This stepwise procedure resulted in the elution of RNA-polymerase B activity as a narrow peak (500 ml), while RNA-polymerase C remained bound. The pooled fractions with RNA-polymerase B activity were diluted to yield less than 0.1 M $(NH_4)_2SO_4$ and subjected to affinity chromatography on DNA-agarose (size 4 × 15 cm, standard buffer equilibration). Loading, washing with at least 4 volumes of standard buffer, and elution with a step to 0.5 M $(NH_4)_2SO_4$ in standard buffer were carried out at a flow rate of 40 ml/h. Again the stepwise procedure was preferred, as gradient elution resulted only in augmenting the volume of the eluted activity. The pooled active fractions were concentrated by adsorption on and elution from 3 ml DEAE (wet settled volume). The concentrated crude enzyme was further purified by gel-exclusion chromatography (Sephadex G-150, 4 × 100 cm). The enzymically active fractions were subjected to a second DEAE-cellulose chromatography (1 × 50 cm). Elution with 200 ml of a linear gradient of 0–0.5 M $(NH_4)_2SO_4$ in standard buffer. Apart from RNA-polymerase activity all fractions were assayed for Zn (fig.1).

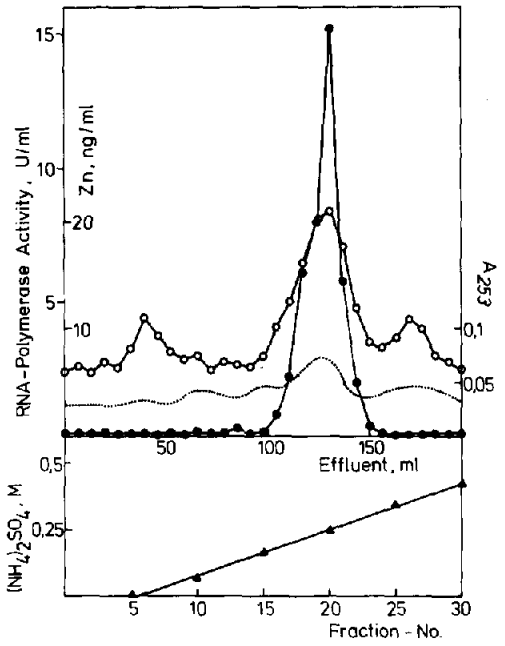

Fig.1. Second DEAE-cellulose chromatography. The column (1 × 50 cm) was loaded with 4 mg protein, washed with 100 ml 50 mM Tris-HCl buffer, pH 7.8, 0.1 mM EDTA, 7 mM β-mercaptoethanol, and 15% (v/v) glycerol, and eluted with a 200 ml linear gradient, 0–0.5 M $(NH_4)_2SO_4$ in buffer (●——●) RNA-polymerase activity, (○——○) zinc content, (. . . .) A_{253}.

Active fractions were pooled and concentrated on 0.5 ml DEAE-cellulose as described above and the concentrated solution (4 ml) was dialysed (2 × 200 ml). Total yield of enzyme protein was 2–5 mg/kg of yeast. The enzyme was stored at −15°C. Lower temperatures resulted in loss of activity. The enzyme proved disc-electrophoretically homogeneous (fig.2).

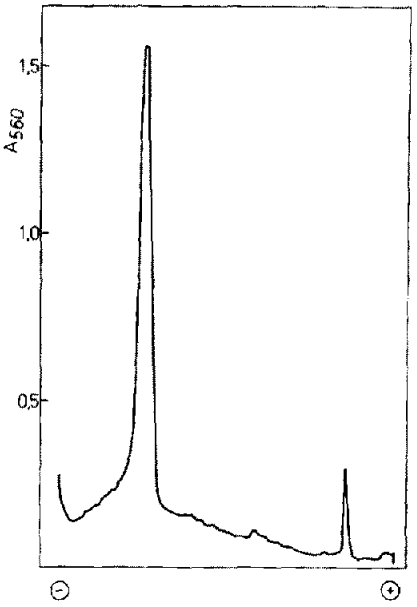

Fig.2. Analytical polyacrylamide gel discelectrophoresis of RNA-polymerase B. Spacer gel 2.5%, separation gel 6% acrylamide. Pre-electrophoresis at 1 mA, 130 V for 1 h. The sample (4.9 μg) was electrophoresed at 1.5 mA per gel stick (5 × 80 mm) and 130–170 V for 2 h. Gels were stained overnight with Coomassie Blue, destained with acetic acid/methanol/water 1:6:14 and recorded on a Pye Unicam SP 1800 spectrophotometer with a SP 1809 scanning device, slit 0.2 mm vertical, 1.5 mm parallel to the gel.

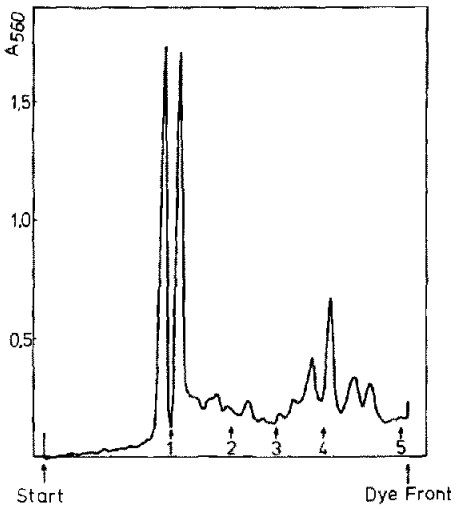

Fig.3. SDS-electrophoresis of RNA-polymerase B. 11.3 μg protein in 10 μl dialysis buffer were mixed with 10 μl 200 mM sodium phosphate buffer, pH 7.2, containing 8 M urea, 2% SDS, 0.01% bromothymol blue, and 2% mercaptoethanol, and were incubated at 45°C for 1 h. The mixture was electrophoresed in a 7.5% acrylamide slab gel (length: 7 cm) made up in 100 mM sodium phosphate buffer, pH 7.2 (with 0.1% SDS in the gel and in the cathode buffer) for 1.5 h at 100 V and 200 mA. The numbered arrows indicate the positions of the following reference substances: (1) γ-globulin (very faint band), (2) phosphorylase a, (3) catalase, (4) carbonic anhydrase, (5) cytochrom c. For staining (at least 48 h), destaining and recording see legend to fig.2.

The specific activity was between 300 and 400 U/mg and agreed with earlier data [15]. SDS-electrophoresis revealed a subunit pattern identical with other work [15,25] except for the additional subunit of 2300 daltons. The following subunits with the indicated stoichiometry were found (fig.3): 170 000(1), 150 000(1), 41 000(1), 28 000(2), 23 000(1) and 19 000(1), giving a mol. wt. of 460 000.

3.2. *Evidence for zinc protein character of RNA-polymerase B*

During purification, a zinc peak paralleled the RNA-polymerase activity. All enzyme preparations examined contained tightly bound Zn^{2+}, which could not be removed by dialysis or by the chromatographic steps of the purification. For 8 different preparations, a 1:1 stoichiometry (0.98 ± 0.4) was found, assuming a mol. wt. of 460 000. This is a lower Zn^{2+} content as reported elsewhere [8,14] for RNA-polymerases from other sources. This might originate from differences in preparation, homogeneity and determinations of zinc and protein. To elucidate the correct stoichiometry, samples of the enzyme were exposed to a tenfold excess of Zn^{2+} in a N_2-gassed buffer lacking EDTA and mercaptoethanol. When, after incubation for 0.5 and 1 h at 4°C and 37°C, excessive Zn^{2+} was removed by gel filtration (Sephadex G-25), the stoichiometry was found to have moved nearer to 1:1 as before. Thus, no Zn^{2+} seems to bind non-specifically to the enzyme.

0.6 mM 1,10-phenanthroline inhibited the enzymic activity by 50% in a way similar to that found for other zinc enzymes (fig.4). 7,8-benzoquinoline of identical structure, except for one N being substituted by a C atom had no chelating ability and was

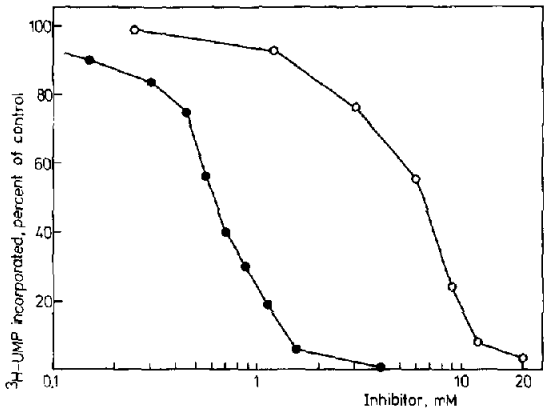

Fig.4. Inhibition of RNA-polymerase B by 1,10-phenanthroline (•——•) and 7,8-benzoquinoline (○——○). 4.9 μg protein were tested for RNA-polymerase activity in the standard assay mixture with the indicated concentrations of inhibitor. Before adding the other assay components, enzyme and inhibitor were mixed and incubated for 1 h at 4°C. Controls contained water instead of inhibitor solution.

ineffective even at a molarity ten times higher.
To exclude inhibitory effects by trapping essential Mn^{2+} in the assay mixture, we studied the reversibility of the phenanthroline-induced inhibition by adding both Mn^{2+} and Zn^{2+}. Whereas Zn^{2+}, present in equimolar concentration as the inhibitor, led to 70% reconstitution of enzymic activity, Mn^{2+} had no effect, even when present in a two-fold excess over the inhibitor. Complete removal of Zn^{2+} from the enzyme was not possible due to irreversible structural changes of the protein portion. Treatment of the enzyme with 1000-fold excess of phenanthroline for 7.5 h, resulted only in loss of half the original zinc content; the loss of zinc was paralleled by an equal loss of specific activity.

The above findings, specific inhibition by 1,10-phenanthroline, stoichiometry, relationship between zinc content and specific activity, and absence of unspecific binding of Zn^{2+}, together with an isolation procedure carefully avoiding metal contaminations, give evidence, that RNA-polymerase B from saccharomyces cerevisiae is a zinc protein, as Valenzuela [14] claimed for all nucleotidyltransferases.

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