DNA DEPENDENT RNA POLYMERASE FROM RAT LIVER MITOCHONDRIA

R. GALLERANI, C. SACCONE, P. CANTATORE and M.N. GADALETA
Institute of Biological Chemistry, University of Bari, 70126 Bari, Italy

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1. Introduction

The purification of mitochondrial DNA-dependent RNA polymerase from rat liver and other organisms appears to be particularly difficult since the enzyme is readily inactivated by a wide range of extraction procedures [1, 2]. We recently succeeded in solubilizing the enzyme from rat liver mitochondria using the detergent deoxycholate [3]. Stabilization of the enzyme activity after extraction was achieved with large concentrations of dithiothreitol and glycerol [3]. Mitochondrial rat liver RNA polymerase solubilized either by sonication or by deoxycholate appears to belong to the bacterial class of RNA polymerases since is inhibited by rifampicin and insensitive to α-amanitin [3, 4].

A mitochondrial RNA polymerase has recently been purified from sonicates of Neurospora mitochondria by repeated glycerol gradient centrifugation [5]. Solubilization and partial purification of mitochondrial enzyme from wild type yeast has been reported by Tsai et al. [6].

In this paper we report the first attempt to purify a mitochondrial RNA polymerase from animal cells. Partial purification of the enzyme was achieved by a procedure including lysis at high ionic strength, DNase treatment, ammonium sulphate fractionation and DEAE-Sephadex A-25 column chromatography.

2. Materials and methods

Male albino rats weighing about 200 g and starved overnight were used in all experiments. Mitochondria were isolated from rat liver under sterile conditions as previously described with special care taken to eliminate nuclear contamination [7].

To solubilize the enzyme, intact mitochondria (about 550 mg of protein) were suspended in buffer containing 25 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DDT), 0.3 M ammonium sulphate pH 7.4, then were lysed by adding sodium deoxycholate at 1.5% final concentration. 25% Glycerol was then added. Lysed mitochondria were centrifuged at 105,000 *g* for 60 min in the Spinco Model L ultracentrifuge. The supernatant was passed through a Sephadex G-25 column (2.8 × 45 cm) in order to remove the sodium deoxycholate. The column was equilibrated with buffer containing 25 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 25% glycerol, 0.2 M KCl. This buffer is called B_{1k}. Flow rate was 0.5 ml/min. This enzyme preparation is reported in the text as DOC extract.

In order to detach the enzyme from the DNA, an aliquot of DOC extract (about 135 mg of protein) was passed through a Sephadex G-25 column (1.8 × 32 cm) equilibrated with buffer B_1 (25 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 5% glycerol). The peak of proteins was incubated with DNase (Worthington, E.C 3.1.4.5) at a final concentration of about 50 μg/ml for 45 min at 0°. Solid ammonium sulphate was then added up to a final concentration of 0.24 g/ml with a few drops of 1 M NaOH in order to keep a constant pH of 7.4.

The ammonium sulphate suspension was centrifuged at 80,000 *g* for 30 min at 4°. The supernatant was passed through a Sephadex G-25 column (2.2 × 39 cm) equilibrated with Buffer B_{2k} (25 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT,

0.1 M KCl, 20% glycerol) in order to remove the ammonium sulphate. The peak containing activity was adsorbed on a DEAE-Sephadex A-25 column (1 X 12 cm) equilibrated with buffer B_{2k}. The column was then eluted with 100 ml of a linear gradient 0.1–1 M KCl in Buffer B_{2k}. 1 ml fractions were collected. Mitochondrial RNA polymerase activity was measured as described before [3] using *E. coli* K_{12} DNA as template. Protein was estimated by the Lowry method [8]. α-Amanitin was purchased from Boehringer, Ingelheim. Rifampicin was kindly given by Lepetit, Milano, Italy.

3. Results

The procedure of purification of mitochondrial RNA polymerase from rat liver is summarized in fig. 1. The only means of detaching mitochondrial DNA from the enzyme was the treatment with DNase, since other techniques such as treatment with streptomycin sulphate or sonication did not give reproducible results. It must be stressed that after the DNase treatment the enzyme activity became rather unstable so that all the successive steps were carried out without interruption.

Results from a typical purification are shown in table 1. After ammonium sulphate fractionation there is a great loss of total enzyme activity. This is due to the fact that part of enzyme, still attached to DNA, remains in the precipitate after addition of ammonium sulphate. The increase in the specific activity starting with mitochondria swollen by P_i is about 800-fold.

The properties of the DOC extract are reported in our previous papers [3]. The supernatant after ammonium sulphate fractionation passed through a Sephadex G-25 column was assayed for the α-amanitin and rifampicin sensitivity at concentrations of 0.15 and 10 μg/ml of the drugs, respectively. The enzymatic activity was unaffected by α-amanitin and inhibited by rifampicin to about 50%. The DNA dependence at this step varied from 50 to 80% in 8 experiments. The remaining DNA independent activity was almost unaffected by DNase showing that this activity was probably not due to DNA bound-enzyme but to some other incorporating activity not DNA dependent. The two different activities could be separated

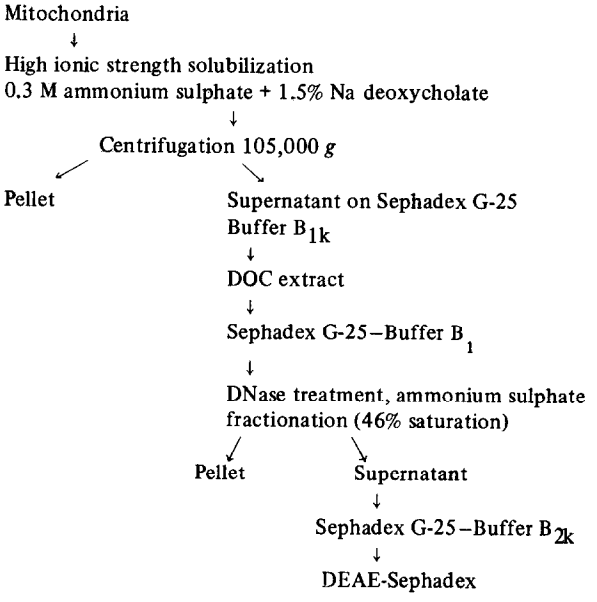

Fig. 1. Purification scheme of RNA polymerase from rat liver mitochondria. Buffer compositions and technical details are reported in Materials and methods.

through the DEAE-Sephadex A-25 column as shown in fig. 2. The fractions corresponding to the first peak excluded from the column (fig. 2) contained a DNA-independent activity. DNase sensitivity of these fractions was checked by incubating in standard conditions 30 μl of each fraction in the presence of 50 μg/ml DNase without adding any external template. No difference was found in the incorporation in the presence or absence of DNase.

The second peak was eluted at about 0.4 M KCl and contained an activity which was DNA dependent (70–100% in 5 different experiments), rifampicin sensitive and resistent to α-amanitin. Thus we conclude that activity of peak II represents the true mitochondrial RNA polymerase activity from rat liver.

In order to calculate the protein concentration of peak II, the corresponding fractions were pooled and dialyzed against a buffer containing 25 mM Tris-HCl pH 7.4 and 5 mM $MgCl_2$. The dialysate was then concentrated and the protein assayed with Lowry's method. (See also legend of fig. 2.)

The specific activity of this peak ranged from 7,000–10,000 pmoles of UMP incorporated per mg of protein in 5 different experiments.

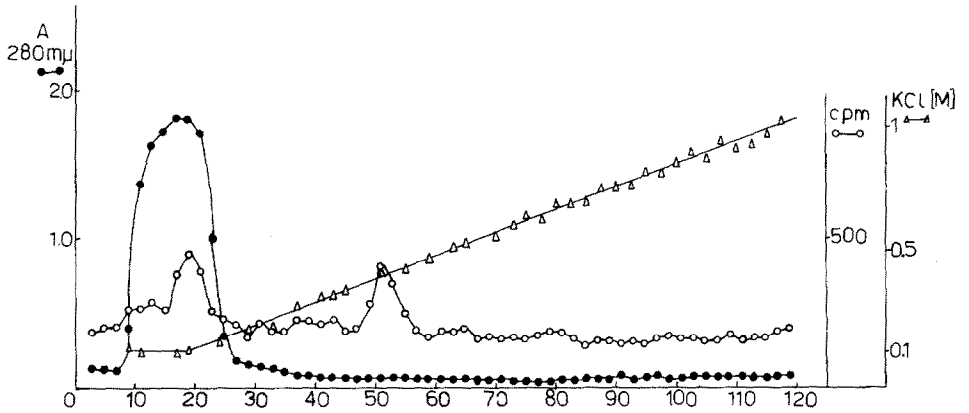

Fig. 2. Chromatography of mitochondrial RNA polymerase on DEAE-Sephadex A-25. The supernatant, after ammonium sulphate precipitation purified through a Sephadex G-25 column (see Materials and methods) was adsorbed on a (1 × 12 cm) DEAE-Sephadex A-25 column equilibrated with B_{2k} buffer. The column was eluted with a linear gradient: 50 ml of 0.1 M KCl in B_{2k} + 50 ml of 1 M KCl in B_{2k}. Volume of each fraction was 1 ml. The enzymatic activity was measured by incubating 30 μl aliquots in standard conditions (see Material and methods). One unit of enzyme is that amount which incorporates one pmole of ^{3}H-UMP per 10 min at 30°. In order to estimate the amount of protein present in the second peak the fractions from 47 to 57 were collected and dialyzed against a buffer containing 25 mM Tris-HCl pH 7.4 and 5 mM $MgCl_2$. They were then concentrated and assayed with the Lowry method [8].

4. Discussion

Mitochondrial DNA-dependent RNA polymerase from rat liver has been purified about 800-fold with the above reported procedure. The possibility that RNA synthetic activity measured in these experiments and attributed to mitochondrial enzyme is actually due to nuclear enzyme can be ruled out by the fact that the enzyme is resistant to α-amanitin and sensitive to rifampicin. This excludes a contamination either from polymerase I or polymerase II of nuclear origin. On the other hand, the enzyme activity cannot be due to bacterial contamination since mitochondria were prepared under sterile conditions as previously reported [7].

From the properties of the purified enzyme we can conclude that mitochondrial RNA polymerase from rat liver resembles bacterial type polymerases. This is in agreement with the data reported by Kuntzel et al [5] with an electrophoretically pure mito-

Table 1
Purification steps of mitochondrial RNA polymerase from rat liver.

Purification steps	Total volume (ml)	Protein (mg)	Total activity	Specific activity	Recovery (%)
1) Mitochondria	15	550	6,200	11.3	100
2) DOC extract	45	520	6,880	13.2	110
3) Supernatant after ammonium sulphate fractionation	16	13.3	665	50.0	43*
4) DEAE-Sephadex (peak II)	11	0.018	154	8,500	10*

* The recovery of these steps was calculated taking into account that only 1/4th of the DOC extract was further processed. The starting activity was measured on swollen mitochondria prepared as reported elsewhere [7]. Treatment with DNase and following steps were performed on 1/4th of the DOC extract as described in Materials and methods. The specific activity is expresses as pmoles of ^{3}H-UMP incorporated in 10 min per mg of protein.

chondrial RNA polymerase from Neurospora. These authors, however, suggest that with its primitive structure the mitochondrial enzyme resembles more the bacteriophage T7 specific enzyme than *E. coli* polymerase since both phage and mitochondrial enzymes consist of a single subunit of relatively low molecular weight [5].

The rat liver mitochondrial enzyme after DEAE-Sephadex A-25 column is readily inactivated and better stabilization conditions must be found in order to study and characterize its structure and function.

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