THE EFFECT OF α-AMANITIN ON RNA SYNTHESIS IN RAT LIVER MITOCHONDRIA

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1. Introduction

In a previous paper [1] we have demonstrated that the mitochondrial RNA polymerase from rat liver is sensitive to rifampicin. Inhibition by rifampicin was dependent on the amount of the enzymic protein in the assay mixture suggesting that the inhibition occurs at the enzyme level as in bacterial systems. In this property mitochondrial RNA polymerase thus resembles the bacterial polymerase and differs from the nuclear one. On the other hand it is well known that α-amanitin in eukariotic cells is a specific inhibitor of Mn^{2+}-$(NH_4)_2SO_4$ activated nuclear RNA polymerase whereas it has no effect on nucleolar polymerase and on bacterial RNA polymerase [2, 3]. In this respect we thought it would be interesting to test the sensitivity of the mitochondrial enzyme towards α-amanitin in order to emphasize the dissimilarity between the nucleoplasmic and the mitochondrial RNA polymerase and at the same time the resemblance of the mitochondrial enzyme with the bacterial one. We have thus investigated the effect of α-amanitin on RNA synthesis in isolated mitochondria as well as in the presence of a solubilized preparation of mitochondrial RNA polymerase from rat liver. The results obtained demonstrate that the mitochondrial enzyme differs from the nucleoplasmic enzyme in sensitivity to α-amanitin.

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 [4]. Swollen mitochondria were obtained by incubating intact mitochondria with 0.1 M sterile phosphate buffer pH 7.4, for 15 min at 30° and then centrifuged. Pellets were collected in B_{1k} buffer. Its composition is described below.

To solubilize the enzyme, intact mitochondria were suspended in buffer containing 25 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), then were lysed by adding sodium deoxycholate at 1.5% final concentration. 25% glycerol and 0.3 M ammonium sulphate pH 7.4 were then added. Lysed mitochondria were centrifuged at 105,000 *g* for 70 min in the Spinco Model L ultracentrifuge. The supernatant was passed through a Sephadex G-25 column (2.8 × 40 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 dithiothreitol, 25% glycerol, 0.2 M KCl. This buffer was called B_{1k}. Flow rate was 0.5 ml/min. This enzyme preparation is reported in the text as DOC extract. All procedures were carried out in the cold.

Nuclei were isolated and purified according to the procedure of Blober and Potter [5].

Mitochondrial RNA polymerase activity was measured as described before [4]. The incubation mixture contained: 53 mM Tris-HCl pH 7.4, 40 μg/ml pyruvate kinase, 64 mM KCl, 4 mM phosphoenolpyruvate, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1 mM each GTP, CTP, ATP, 0.1 mM ^{3}H-UTP, specific activity 1 Ci/mmole (BioSchwarz, New York) 4% glycerol, 0.2 mM DTT.

The reaction was started by adding either mitochondria or solubilized enzyme at the concentrations given in the figures. After 10 min incubation at 30° the reaction was stopped by adding 5 ml of 5% trichloroacetic acid containing 1/10 of saturated sodium pyrophosphate solution. The tubes were cooled in ice

for a few min and treated further as described earlier [4].

Protein was estimated by the Biuret method.

α-Amanitin was in part purchased from Boehringer, Ingelheim and the rest a gift of Prof. Stirpe.

3. Results and discussion

The effect of various concentrations of α-amanitin on RNA synthesis in the presence of isolated nuclei or mitochondria from rat liver is reported in fig. 1. The results clearly demonstrate that concentrations of α-amanitin which cause 80% inhibition of nuclear RNA-polymerase do not affect the mitochondrial enzyme.* High concentrations of the drug inhibit the synthesis of RNA in the mitochondria but to a lesser extent (34%) as compared to nuclei.

To investigate better the effect of α-amanitin on mitochondrial RNA polymerase we have tested the drug on solubilized enzyme. Solubilization was achieved by using the detergent deoxycholate in the presence of dithiothreitol and glycerol as protecting agents. After passage through a Sephadex G-25 column the enzymic activity was assayed as previously described in the presence of external added DNA. Freshly prepared, solubilized enzyme, displayed only 20–30% DNA dependence but was completely inhibited by DNase or actinomycin D, thus indicating that the RNA synthesis activity is due to DNA-dependent RNA polymerase and not to some other enzymic activities. The results summarized in fig. 2 demonstrate that up to 1 μg/ml of α-amanitin does not inhibit mitochondrial RNA polymerase even in the presence of small amounts of protein. Higher concentration causes an extent of inhibition which is dependent on the amount of enzyme present in the incubation mixture. The possibility that RNA synthetic activity measured in these experiments and attributed to mito-

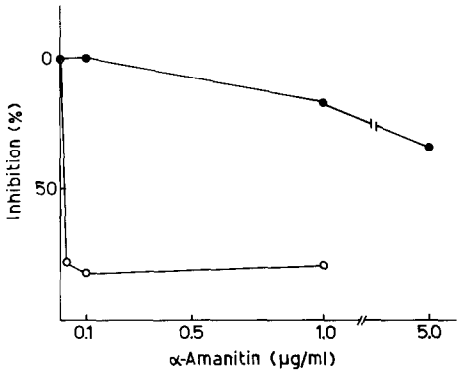

Fig. 1. Effect of α-amanitin on RNA synthesis of swollen mitochondria and nuclei from rat liver. Mitochondrial RNA polymerase activity was assayed as described in Materials and methods, using 0.225 mg of mitochondrial protein in 0.15 ml of incubation mixture. Nuclear Mn^{2+}-$(NH_4)_2SO_4$ activated RNA polymerase was assayed as reported by Jacob et al. [3] except that the specific activity of ^{3}H-UTP in the incubation mixture was 25 μCi/μmole. In a final volume of 0.4 ml 0.6 mg of nuclear protein were incubated for 40 min at 37°. α-Amanitin was added to the incubation mixture to reach the final concentration shown in the figure. 100% mitochondrial activity was equivalent to 11.3 ± 0.5 pmoles of UMP incorporated/mg protein/10 min. 1 pmole corresponds to 165 cpm. A blank of 260 ± 30 cpm obtained from a zero incubation time assay mixture, was subtracted from all values. 100% nuclear activity was 1,280 pmoles/mg protein/40 min. 1 pmole corresponds to 5.5 cpm. The values reported are derived from the means of 10 experiments for mitochondria and 4 for the nuclei. ○—○ Nuclei, ●—● Mitochondria.

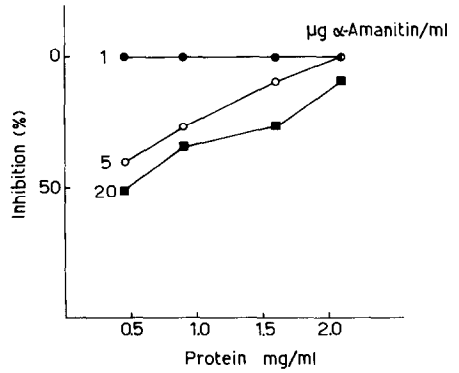

Fig. 2. Effect of α-amanitin on solubilized mitochondrial RNA polymerase at various enzyme concentrations. Increasing amount of DOC extract were used in the conditions reported in Materials and methods. 100% of DOC extract activity was equivalent to 13.2 ± 1.4 pmoles UMP incorporated/mg protein/10 min.

* In a first series of experiments we found that α-amanitin stimulated mitochondrial RNA synthesis probably by inhibiting the nuclease activity present in the mitochondrial preparation. Further experiments, done with several other batches of α-amanitin, made us aware that the action on nuclease activity and therefore on mitochondrial RNA-polymerase was due to some contaminant, whose nature and origin is unclear, present in the α-amanitin solution.

chondrial enzyme is actually due to the Mg^{2+}-activated nucleolar enzyme contamination can be ruled out by the fact that the enzyme activity is inhibited by rifampicin and, when essayed in the presence of intact isolated organelles, is also sensitive to atractyloside which, by inhibiting the passage of ATP across the inner mitochondrial membrane, is a useful tool to distinguish between the nuclear and the mitochondrial polymerase activity [6]. Our results clearly show that DNA-dependent RNA polymerase from rat liver mitochondria is not affected by the concentrations of α-amanitin which inhibit nucleic RNA polymerase. Therefore we can conclude that in this property mitochondrial RNA polymerase in rat liver is different from the Mn^{2+}- $(NH_4)_2SO_4$ activated nuclear polymerase. On the other hand, the sensitivity of the enzyme to rifampicin indicates that it differs from the RNA polymerase present in nucleoli of higher organisms whereas it resembles the bacterial polymerase. The insensitivity to α-amanitin of mitochondrial enzyme from rat liver is in agreement with the data recently reported by M.J. Tsai et al. using the DNA-dependent RNA polymerase from yeast mitochondria [7]. In contrast the yeast enzyme seems not to be inhibited by rifampicin [7] whereas the rat liver enzyme is rifampicin sensitive. A similar controversy exists concerning the effect of rifampicin on the RNA polymerase activity of chloroplasts [8]. In our opinion the discrepancy could be due to the different experimental conditions and preparation procedures used by various authors or could mean a real difference in the enzymes from different sources.

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