AMANITIN BINDING TO CALF THYMUS RNA POLYMERASE B

M. MEIHLAC*, C. KEDINGER and P. CHAMBON
Institut de Chimie Biologique, Faculté de Médecine, Strasbourg, France

and

H. FAULSTICH, M.V. GOVINDAN and T. WIELAND
Max-Planck-Institut für Medizinische Forschung, Abteilung Chemie, Heidelberg, Germany

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1. Introduction

We have previously reported that α-amanitin specifically inhibits calf thymus RNA polymerase B [1]. It was shown (a) that inhibition by α-amanitin is dependent on the amount of enzyme present and independent on the amount of DNA and nucleoside triphosphates; (b) that calf thymus RNA polymerase A and *E. coli* RNA polymerase are not inhibited; (c) that α-amanitin is effective at very low concentration and that a very limited number of α-amanitin molecules, probably only one [2], is necessary to inhibit one molecule of RNA polymerase B. All these findings suggest a direct interaction between enzyme and amanitin.

Using ^{14}C-methyl-γ-amanitin, we have now obtained evidence that RNA polymerase B forms a very stable complex with amanitin.

2. Material and methods

Calf thymus RNA polymerase B was solubilized and purified as described elsewhere [2]. Two enzyme fractions were used in the present experiments: fraction BII which corresponds to the phosphocellulose column step and fraction BIII which corresponds to the hydroxyapatite column step. Fractions BII and BIII were estimated to be 5-10% and 30-40% pure, respectively.

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The reaction mixture (0.25 ml) contained 100 mM tris-HCl, pH 7.9, 3 mM Mn^{2+}, 1 mM each nucleoside triphosphate, 4 mM thioglycerol and 70 μg calf-thymus DNA. Fractions BII and BIII were assayed in the presence of 0.048 M and 0.096 M ammonium sulphate, respectively. Under these conditions, the specific activities (nmole ^{32}P-GMP incorporated in 10 min at 37°

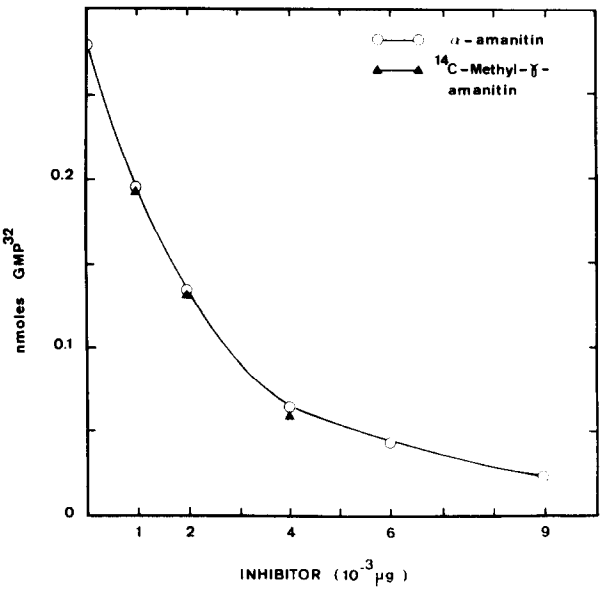

Fig. 1. Comparison of the inhibitory effects of α-amanitin and ^{14}C-methyl-γ-amanitin. Incubation conditions were as described in Material and methods. Inhibitors were added to reaction mixture before the enzyme (2 μg of fraction BIII).

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per mg protein) of fractions BII and BIII were 20 and 140, respectively. Incubations were processed as previously described [1].

γ-Amanitin was labeled by *O*-methylation of the phenolic group using ^{14}C-methyliodide in acetone in the presence of K_2CO_3 [3]. The specific activity of ^{14}C-methyl-γ-amanitin was 1,5 × 10^5 dpm per μg.

3. Results and discussion

γ-Amanitin is even more toxic than α-amanitin *in vivo* [4] and its 6-methyl derivatives exhibit an amanitin-like action [3]. However their *in vitro* effects upon RNA polymerase B were unknown. Fig. 1 shows that, *in vitro*, methyl-γ-amanitin was as inhibitory as α-amanitin.

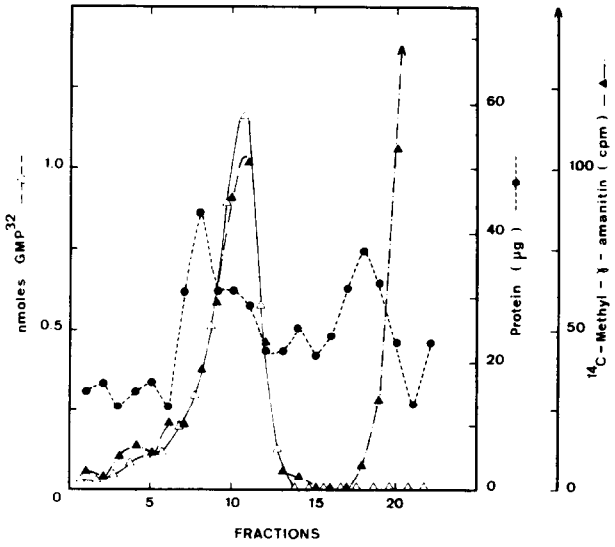

Fig. 2. Determination of the binding of ^{14}C-methyl-γ-amanitin to enzyme B (fraction BII) on a glycerol density gradient. 570 μg of fraction BII was incubated for 2 min at 37° with 0.016 μg of labeled methyl-γ-amanitin in 0.25 ml of 10 mM tris-HCl, pH 7.9, 2 mM $MnSO_4$, 10% glycerol. The mixture was layered on 4.3 ml of 10-30% glycerol gradient containing 50 mM tris, pH 7.9, 2 mM $MnSO_4$, 0.1 mM EDTA and 0.1 mM dithiothreitol. The gradient was centrifuged at 50,000 rpm in a Spinco SW-50 rotor for 5 hr at 0°. Fractions, 0.2 ml each, were collected through a hole in the bottom of the tube. The bottom of the tube is on the left. Enzyme activity was measured as described in Material and methods and is expressed as nmole of ^{32}P-GMP incorporated in 10 min. Proteins were determined according to Lowry.

To determine whether ^{14}C-methyl-γ-amanitin can form a stable complex with the enzyme, a mixture of ^{14}C-methyl-γ-amanitin and RNA polymerase B (fraction BII) was incubated and sedimented through a

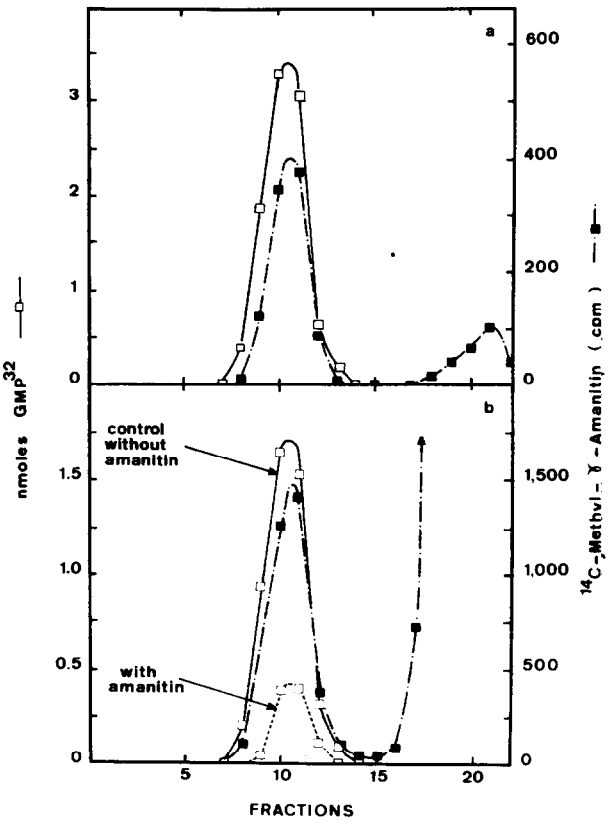

Fig. 3. Cosedimentation of ^{14}C-methyl-γ-amanitin and enzyme B (fraction BIII) on glycerol density gradients.

a) excess enzyme: 106 μg of fraction BIII was incubated for 10 min at 37° with 0.01 μg of labeled methyl-γ-amanitin in the buffer described in fig. 2. The mixture was layered on 4.3 ml of a 15 to 30% glycerol gradient containing 50 mM tris-HCl, pH 7.9, 2 mM $MnSO_4$, 0.1 mM EDTA, 0.1 mM dithiothreitol and 50 mM ammonium sulphate. The gradient was centrifuged at 65,000 rpm in a Spinco SW-65 rotor for 4 hr at 0°. Fractions were collected as described in fig. 2. Enzyme activity was measured as described in Material and methods and is expressed as nmole of ^{32}P-GMP incorporated in 10 min at 37°.

b) excess ^{14}C-methyl-γ-amanitin: 53 μg of fraction BIII was incubated for 10 min at 37° with 1.4 μg of labeled methyl-γ-amanitin. The glycerol density gradient and the conditions of enzyme assay were as described above. Enzyme activity in the absence of inhibitor was determined in a parallel run.

glycerol density gradient. As shown in fig. 2, there was a peak of radioactive ^{14}C-methyl-γ-amanitin in the same position as the peak of RNA polymerase activity. The radioactivity present at the top of the gradient corresponds to free ^{14}C-methyl-γ-amanitin. The binding of methyl-γ-amanitin to enzyme B appears to be very specific since there was no radioactivity bound either to other proteins present in the partially purified fraction BII (fig. 2), to *E. coli* RNA polymerase, or to calf thymus RNA polymerase A (results not shown).

The fact that there was no tailing of radioactivity above the peak of bound methyl-γ-amanitin suggests that a rather stable complex was formed. This stability was confirmed by experiments performed with a more purified fraction (fraction BIII). Fig. 3a shows that under conditions of enzyme excess most of the ^{14}C-methyl-γ-amanitin was bound to the enzyme. On the other hand, with excess methyl-γ-amanitin most of the RNA polymerase activity was inhibited (fig. 3b). In the latter case the observed inhibition (80%) was accounted for by the amount of ^{14}C-methyl-γ-amanitin bound to the enzyme, assuming that the stoichiometry of the binding is 1:1 and the enzyme 30-40% pure [2]. Therefore there is no doubt that the formation of the complex is responsible for the inhibition of RNA synthesis. Since α-amanitin [1] as well as methyl-γ-amanitin [2] can prevent any further elongation of growing RNA chains, studies are now in progress to determine whether ^{14}C-methyl-γ-amanitin binds to RNA polymerase B engaged in a transcription complex (enzyme-DNA-RNA complex) and whether DNA and/or RNA are then released from the complex.

Acknowledgements

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