α-AMANITIN : A SPECIFIC INHIBITOR OF ONE OF TWO DNA-DEPENDENT RNA POLYMERASE ACTIVITIES FROM CALF THYMUS

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Received November 7, 1969

SUMMARY. - α-amanitin selectively inhibits RNA synthesis catalyzed by calf thymus RNA polymerase activity B by interacting with the enzyme and inhibiting chain elongation.

α-amanitin, a powerful toxin of the toadstool Amanita phalloides (1), was previously reported to inhibit RNA synthesis both *in vivo* and in liver nuclei *in vitro* (2,3). During the course of purification of DNA-dependent RNA polymerase from calf thymus we found that α-amanitin was a very efficient and highly specific inhibitor of one of the two RNA polymerase activities present in calf thymus. This very interesting and promising property of α-amanitin prompted us to study the mechanism of its inhibitory effect.

MATERIAL AND METHODS

RNA polymerase activities A and B of calf thymus were solubilized by sonication and separated by ammonium sulphate fractionation and chromatography on DEAE cellulose, A being eluted at 0.15 M ammonium sulphate and B at 0.30 M (manuscript in preparation). Fraction B was further purified on hydroxyapatite column. In the present experiments specific activities of fractions A and B were respectively 2.8 and 9.4 mμmoles of GMP^{32} incorporated/10 min/mg protein. These values corresponded to about a 30-fold

purification for enzyme A and a 100-fold purification for enzyme B. E. coli RNA polymerase was purified, with minor modifications, according to Chamberlin and Berg (4) and its activity was 700 mμmoles GMP^{32} incorporated/mg protein/10 min.

Incubation conditions were as follows, in a final volume of 0.25 ml : tris HCl pH 7.9 80 mM, $MnSO_4$ 2 mM, CTP, UTP, ATP, (α-P^{32}) GTP (1-2 mC/mmole) 0.5 mM, calf thymus DNA (Worthington) 60 μg, ammonium sulphate 32 mM, thioglycerol 20 mM. Unless otherwise specified, RNA synthesis was initiated by addition of enzyme (usually in 0.10 ml of tris HCl 50 mM pH 7.9, thioglycerol 50 mM and glycerol 25 °/₀). The samples were incubated for 10 min at 37°C, then 10 °/₀ TCA containing 0.02 M sodium pyrophosphate was added. The precipitates were collected on Whatman glass filter GF/C, rinsed with TCA-pyrophosphate, dried and counted.

α-amanitin was supplied by Dr. T. Wieland.

RESULTS

Figure 1 shows that RNA synthesis by RNA polymerase activity B was inhibited by α-amanitin at very low concentrations of toxin. On the contrary RNA polymerase activity A and E. coli RNA polymerase were not inhibited even at concentrations of α-amanitin 1000-fold higher. It is interesting to note that mixing enzyme A and B in the presence of the lowest concentration of α-amanitin (0.004 μg/0.25 ml) resulted in the expected inhibition suggesting that lack of inhibition of enzyme A is not due to preferential binding of α-amanitin by some component present in enzyme preparation A. Increasing the ionic strength did not modify the effect of α-amanitin on RNA polymerase activities A and B, indicating that lack of inhibition of RNA polymerase A is either an intrinsic property of the enzyme or, less probably, related to a very tight

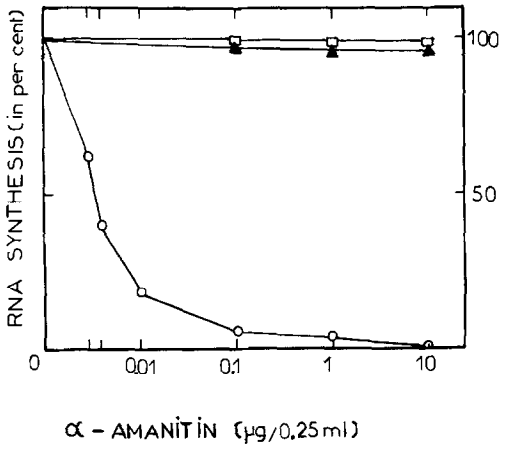

Figure 1. - Comparative effect of α-amanitin on calf thymus RNA polymerase A (—▲—) and B (—○—) and on E. coli RNA polymerase (—□—).
Incubation conditions were as described in Material and Methods. E. coli enzyme was incubated in the same reaction mixture except that ammonium sulphate was omitted and that $MnSO_4$ was 1 mM and $MgCl_2$ 4 mM. In these assays, α-amanitin was added to reaction mixture before the enzyme. Amounts of enzyme in incubations were 149 µg for calf thymus RNA polymerase activity A, 82 µg for calf thymus RNA polymerase activity B and 2.4 µg for E. coli RNA polymerase.

binding of some component to the enzyme preventing the interaction with α-amanitin, but which in other respects would not affect the enzyme catalytic properties. In any case, these results suggest that α-amanitin inhibits transcription by acting on RNA polymerase rather than on DNA.

This is confirmed by results presented in table 1 and figure 2 which show that, for given amounts of enzyme and α-amanitin, the inhibition was independent of the amount of DNA present in the reaction mixtures (table 1) and that, for a given amount of α-amanitin, the extent of inhibition was related to the amount of

TABLE 1. - Inhibition of calf thymus RNA polymerase B by α-amanitin is independent of DNA concentration.

Incubation conditions	addition of α-amanitin 0.004 µg	mµmoles GMP^{32} incorporated
Standard[+]	-	0.85
Standard	+	0.47
" except that DNA was 90 µg	-	0.78
" except that DNA was 90 µg	+	0.45
" except that DNA was 120 µg	-	0.82
" except that DNA was 120 µg	+	0.46

[+]As described in Material and Methods. RNA synthesis was initiated by addition of 84 µg of enzyme fraction B.

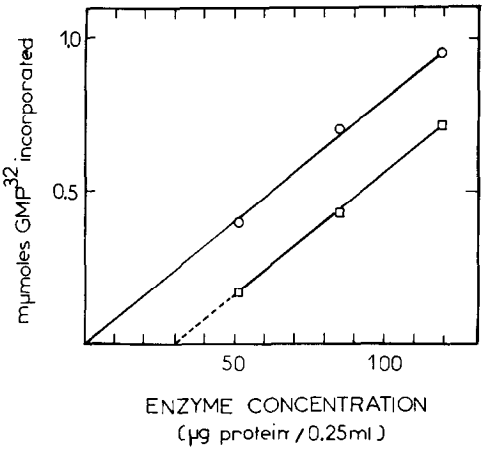

Figure 2. - The extent of inhibition of calf thymus RNA polymerase B brought about by a given concentration of α-amanitin is related to enzyme concentration. Incubation conditions were as described in Material and Methods. RNA polymerase fraction B was added last to reaction mixtures, in the absence (—○—) or in the presence (—□—) of α-amanitin (0.004 µg per 0.25 ml).

enzyme (fig. 2). Extrapolation of curves of figure 2 indicates that 0.004 µg of α-amanitin would completely inhibit the amount

of RNA polymerase B contained in 30 µg of protein of the partially purified preparation. Assuming that the molecular weight of RNA polymerase B is close to that of E. coli RNA polymerase (about 400,000) and taking into account that our enzyme preparation is still not pure, these results would indicate that a very limited number of α-amanitin molecules (M.W. 916), perhaps only one, would be necessary to inhibit one molecule of RNA polymerase B.

Results shown in table 2 indicate that α-amanitin inhibits at least the elongation step of transcription, since pre-incubation of the enzyme with DNA or addition of α-amanitin after polymerization had started did not modify the extent of inhibition (in our incubation conditions not more than 30 per cent of RNA synthesis between 10 and 20 min corresponded to initiation of new RNA chains). It does not seem that there is a competition

TABLE 2. - α-amanitin inhibits RNA chain elongation catalyzed by calf thymus RNA polymerase B.

Order of addition of components to reaction mixture	Incubation time	mµmoles GMP^{32} incorporated
Standard+	10 min	0.850
Enzyme, nucleoside triphosphate, α-amanitin (1 µg), 2 min of pre-incubation, then DNA.	10 min	0.016
Enzyme, DNA, 2 min of pre-incubation, then α-amanitin (1 µg) and nucleoside triphosphate.	10 min	0.023
Standard+	20 min	1.402
Standard+, α-amanitin (1 µg) added after 10 min of incubation.	20 min	0.854

+As described in Material and Methods. 82 µg of enzyme fraction B were added to each incubation.

between α-amanitin and nucleoside triphosphates, because increasing the triphosphate concentration did not change the extent of inhibition.

DISCUSSION

Although we cannot exclude at the present time that α-amanitin could also inhibit previous steps in transcription, our results suggest that α-amanitin acts on calf thymus RNA polymerase activity B as streptolydigin does on bacterial enzyme by inhibiting chain elongation (5).

The fact that E. coli polymerase is not inhibited by α-amanitin is in keeping with a previous report showing that α-amanitin does not inhibit bacterial growth (6). Our results support the hypothesis that the structures of bacterial and animal RNA polymerases are different as already suggested by the lack of effect of rifampicin on various crude animal RNA polymerases (7,8) and on calf thymus RNA polymerase activities A and B (unpublished results). The inability of calf thymus enzymes A and B to use native λ and T_4 phage DNA as template is also consistent with this hypothesis (unpublished results).

Although it is still possible that a more purified fraction of RNA polymerase activity A would become sensitive to α-amanitin, it is tempting to conclude from our results that the specific inhibition of RNA polymerase B reflects a structural difference between the two calf thymus RNA polymerases. Such a difference is also suggested by the elution pattern from DEAE cellulose. That these two RNA polymerase activities could also have specific functions in the cellular transcription is indicated by the work of Stirpe *et al.* (2,3) who reported that RNA synthesis catalyzed by isolated mouse and rat liver nuclei was inhibited by α-amanitin only when incubating at high ionic strength, i.e. in conditions

where RNA polymerase present in the extranucleolar part of chromatin was activated (9). These observations suggest that calf thymus RNA polymerase fraction B corresponds to the extranucleolar RNA polymerase. Very recent results of Roeder and Rutter (10), which were brought to our attention while this manuscript was in preparation, are also in agreement with this interpretation.

Acknowledgements. - We wish to thank Prof. T. Wieland for a generous gift of α-amanitin, and Mrs. G. Alaoui and Mr. G. Dretzen for technical assistance.

J. L. Mandel Jr. is a Fellow of the Ligue Nationale Française contre le Cancer.

F. Gissinger is a Stagiaire de Recherche au CNRS.

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This investigation was supported by grants from the Délégation Générale à la Recherche Scientifique et Technique and the Commissariat à l'Energie Atomique.

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