Studies on the inhibition by α -amanitin of single-step addition reactions and productive RNA synthesis catalysed by wheat-germ RNA polymerase II

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The rate of formation of a single phosphodiester bond with UTP substrate, U-A primer, poly[d(A-T)] template and wheat-germ RNA polymerase II is greatly depressed in the presence of α -amanitin. Half-maximal inhibition occurs at 0.04 μ g/ml, in close agreement with published values for inhibition of productive RNA synthesis with class II RNA polymerases from higher-plant species. However, a sizeable proportion of U-A-U synthesis is resistant to inhibition by excess α -amanitin. In the additional presence of ATP, i.e. under experimental conditions permitting RNA chain elongation, the synthesis of poly[r(A-U)] is arrested after the formation of the first phosphodiester bond. The results support the contention that the main enzymic process disrupted by α -amanitin is the translocation step of the transcription complex along the DNA template.

INTRODUCTION

α -Amanitin, a powerful toxin from the toadstool *Amanita phalloides*, inhibits RNA synthesis catalysed by wheat-germ RNA polymerase II both *in vivo* (Jendrisak, 1980) and *in vitro* (Guilfoyle & Jendrisak, 1978; Jendrisak & Guilfoyle, 1978). Plant RNA polymerases II, like all RNA polymerases II from higher eukaryotes, are typically half-maximally inhibited by α -amanitin at concentrations in the range 10–50 nM (Wieland & Faulstich, 1978; Sentenac, 1985). Cochet-Meilhac & Chambon (1974) have demonstrated that the amatoxin binds very tightly to calf thymus RNA polymerase II ($K_d = 10^{-9}$ M, dissociation half-time of the complex of about 100 h at 0 °C) with a 1:1 stoichiometry. Although it is clearly established that such a tight binding impedes transcription, the mechanisms are not yet fully understood. For instance, Vaisius & Wieland (1982) have reported that purified calf thymus RNA polymerase II catalyses the template-directed synthesis of a single phosphodiester bond with a dinucleotide primer and a single ribonucleoside triphosphate substrate in the presence of a high concentration of α -amanitin. Moreover, under experimental conditions favouring the elongation of RNA, the transcription complex was blocked at initiation (that is after formation of the first phosphodiester bond) by α -amanitin. Vaisius & Wieland (1982) concluded that the translocation step of the transcription complex along the DNA template is the enzymic process blocked by α -amanitin. A variant behaviour has been reported, however, for yeast RNA polymerase II (Lescure *et al.*, 1981), wheat-germ RNA polymerase II (Dietrich *et al.*, 1985) and a HeLa-cell extract (Luse & Jacob, 1987). In all these latter studies, the template-directed reaction of single-step addition of a nucleotide to a dinucleotide primer was strongly inhibited by α -amanitin, suggesting the possibility that the fungal toxin inhibits phosphodiester bond formation.

Taken together, these divergent results may suggest

mechanistic differences in the inhibition process with the enzymes purified from plant, yeast and mammalian cells, or between the purified enzymes and the more complex transcription systems, which, in addition to the RNA polymerase II, do contain the transcription factors. We report here that in transcription studies with U-A as a primer, UTP as substrate and poly[d(A-T)] as template the rate of formation of a single phosphodiester bond by wheat-germ RNA polymerase II, i.e. the synthesis of U-A-U, was greatly depressed in the presence of excess α -amanitin. However, the reaction was not totally inhibited, such that the amount of U-A-U synthesized was nearly stoichiometric with respect to that of enzyme in the transcription assay. Under experimental conditions allowing RNA chain elongation (i.e. in the additional presence of ATP), we found that productive elongation, i.e. the synthesis of poly[r(A-U)], was blocked after U-A-U formation by α -amanitin. Hence wheat-germ RNA polymerase II exhibited the same patterns of inhibition reported for either yeast (Lescure *et al.*, 1981) or calf thymus (Vaisius & Wieland, 1982) RNA polymerase II, and we propose an explanation for the apparently divergent results with these two systems.

MATERIALS AND METHODS

Reagents

Nucleoside triphosphates and the dinucleoside monophosphates were purchased from Sigma Chemical Co. α -Amanitin was from Boehringer Mannheim. [α - 32 P]UTP (410 Ci/mmol, 1 Ci = 3.7×10^{10} Bq) was from Amersham International. Wheat-germ RNA polymerase IIA was purified by the method of Jendrisak & Burgess (1975), as described previously (Dietrich *et al.*, 1985).

Reaction assays

Final volumes were 10 μ l. Incubations were effected at 35 °C for 30 min. Reactions were stopped by mixing the

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10 μ l reaction mixture with 10 μ l of stop solution containing 1 mM-EDTA, 80% formamide and 0.1% Xylene Cyanol. Unless otherwise noted in the Figure legends, the reaction mixtures contained 1.8 μ M-poly[d(A-T)], 0.9 mM-U-A, 18 nM-enzyme, 5 μ M-[α - 32 P]UTP and 1.5 mM-MnCl₂ in transcription buffer as described in Job *et al.* (1987). Portions (5 μ l) were loaded on 20% polyacrylamide/8 M-urea gels (0.03 cm $\times$ 30 cm $\times$ 40 cm). Electrophoresis was conducted at a constant power of 90 W, with a 50 mM-Tris/borate buffer, pH 8.0 (Maniatis *et al.*, 1982), until the marker dye Xylene Cyanol had migrated 15 cm. For quantitative measurements of U-A-U synthesis, portions (2 μ l) were spotted on poly(ethyleneimine)-cellulose sheets, which were developed with 1 M-formic acid/0.1 M-LiCl. All spots containing radioactivity were cut out and counted for radioactivity (Job *et al.*, 1987).

Calculations and curve-fitting

Owing to the structure of the deviation of the observed values (v_i), the data were weighted by $1/v_i^2$ (Wong, 1975) when processed with a program providing an iterative fit to eqns. (1) and (2) in the Results section, by using the Gauss-Newton method (Cleland, 1979). The superiority of eqn. (1) compared with eqn. (2) was assessed by the procedure of Bardsley (1986), by computing the variance ratio F from the square of the residuals by using eqn. (1) versus that with eqn. (2).

RESULTS

Effect of α -amanitin upon formation of U-A-U with U-A primer

In a typical reaction assay, the synthesis of U-A-U occurred after the incubation for 30 min at 35 °C of reaction mixtures prepared under the standard conditions indicated in the Materials and methods section. When analysed by t.l.c., U-A-U migrated with an R_F value of 0.35–0.4, whereas unchanged UTP remained bound at the origin (Fig. 1a, lanes 1). As clearly shown in Fig. 1(a), the formation of this transcription product was absolutely dependent on the presence of enzyme, DNA template, bivalent cation and primer (lanes 2–5). The formation of U-A-U was strongly inhibited by α -amanitin (lane 6). However, the reaction was not totally suppressed by the fungal toxin. The extent of product formation was strongly diminished upon addition of ATP, indicative of elongation to poly[r(A-U)] chains (lane 7). Fig. 1(b) shows the same reaction mixtures as in Fig. 1(a), but analysed by high-resolution gel electrophoresis, with results that are consistent with those obtained by t.l.c. The gel also resolved the poly[r(A-U)] chains synthesized on the poly[d(A-T)] template (lane 7). The low concentration of U-A-U detected under the conditions allowing RNA synthesis is consistent with the results of a previous study (Job *et al.*, 1987), from which we proposed that abortive synthesis normally accompanies productive RNA chain elongation in reactions catalysed by wheat-germ RNA polymerase II, as documented in the case of *Escherichia coli* RNA polymerase (for a review see von Hippel *et al.*, 1984).

The influence of α -amanitin concentration on U-A-U synthesis was assessed by determination of the apparent inhibition constant. The data in Fig. 2(a) show the same pattern of behaviour as observed in Fig. 1, i.e. U-A-U synthesis was diminished but not completely suppressed,

Fig. 1. Radioautographic analyses by t.l.c. and gel electrophoresis of the synthesis of U-A-U with poly[d(A-T)] as template and U-A as primer

Reaction assays were prepared as described in the Materials and methods section with 5 μ M-[α - 32 P]UTP (4.54 Ci/mmol). Samples [2 μ l (a) and 5 μ l (b) (reaction mixtures+stop solutions)] were analysed by t.l.c. on poly(ethyleneimine)-cellulose sheets (a) and by gel electrophoresis (b); radioautography was for 17 h at -70 °C. In (a) ascending development was carried out up to 10 cm above the origin (UTP); the spots labelled U-A-U migrated for 3.5–4 cm. In (b) XC shows the migration of Xylene Cyanol. Lanes 1, complete reaction mixture; lane 2, minus enzyme; lane 3, minus template; lane 4, minus MnCl₂; lane 5, minus primer; lane 6, +1 μ g of α -amanitin/ml (i.e. 1.09 μ M); lane 7, +20 μ M-ATP.

even at 10 μ g of α -amanitin/ml (i.e. 10.9 μ M). The simplest equation fitting these data is eqn. (1):

$$[U-A-U] = P_1 + \frac{P_2}{1 + \frac{[\alpha\text{-amanitin}]}{P_3}} \quad (1)$$

for the values of the best-fit parameters listed in Table 1. Attempts to fit the data in Fig. 2(a) by using a simpler equation containing only two parameters such as eqn. (2) were unsatisfactory, since mathematical convergence could only be obtained with worst sum of the residual squares.

$$[U-A-U] = \frac{P_2}{1 + \frac{[\alpha\text{-amanitin}]}{P_3}} \quad (2)$$

Fig. 2. Effect of α -amanitin on U-A-U synthesis catalysed by wheat-germ RNA polymerase II

(a) Transcription with poly[d(A-T)] template, U-A primer, $5 \mu\text{M}$ - $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (4.54 Ci/mmol), 18 nm -enzyme (as in Fig. 1) and α -amanitin from 0 to $10 \mu\text{g/ml}$. U-A-U synthesis was measured after 30 min at 35°C by t.l.c. The smooth line is a computer fit to eqn. (1) in the Results section for the values of the best-fit parameters listed in Table 1. The Figure shows only part of the data used for curve-fitting (21 data points). In particular, the concentration of U-A-U synthesized in the absence of α -amanitin was $698 \pm 20 \text{ nm}$. (b) Relative residuals for the experimental data (v) in (a) and the simulated data (v_{calc}) with eqn. (1) ($\bullet$) or eqn. (2) ($\circ$) for the values of the best-fit parameters listed in Table 1. (c) Effect of enzyme concentration on the residual synthesis of U-A-U in the presence of $20 \mu\text{g}$ of α -amanitin/ml (i.e. $21.8 \mu\text{M}$). Transcription assays were as in (a) except that enzyme concentration was varied. $\circ$, Standard procedure as described in the Materials and methods section. $\bullet$, The transcription assay mixtures were heated for 7 min at 60°C (Vaisius & Wieland, 1982) before the t.l.c. analysis. All measurements in (a) and (c) were effected relatively to control assays conducted under identical conditions, but lacking the DNA template.

The numerical value of the variance ratio F for the data in Fig. 2(a), calculated by the procedure of Bardsley (1986) (Table 1), as well the distribution of the relative residuals by using eqn. (1) or eqn. (2) (Fig. 2b), give strong evidence for the superiority of eqn. (1) compared with eqn. (2).

From these analyses we conclude that wheat-germ RNA polymerase II catalyses the reaction of single-step addition of a nucleotide to a dinucleotide primer in the presence of excess α -amanitin, though to a much smaller extent than in the absence of the fungal toxin.

Fig. 2(c) shows that the amount of product U-A-U whose formation escaped inhibition by α -amanitin was dependent on the amount of enzyme in the transcription assay. A saturation curve was obtained in the presence of $21.8 \mu\text{M}$ - α -amanitin, with a half-maximal apparent saturation at about 20 nm -enzyme. From these data the calculated occupancy of the poly[d(A-T)] template by the enzyme was of the order of 40 bp. Since the size of the site occupied by the wheat-germ RNA polymerase II on simian-virus-40 DNA is 35–41 bp (Chandler & Gralla, 1980), the results in Fig. 2(c) indicate that complete saturation of the template could be obtained, a finding that renders it unlikely that the observed U-A-U synthesis

in the presence of α -amanitin was simply due to a small amount of contaminating RNA polymerase I and III in the enzyme preparation. Fig. 2(c) also shows that the saturation pattern after heating the reaction mixtures to 60°C for 7 min before the t.l.c. analysis was virtually identical with (but somewhat less scattered than) that seen with the standard procedure described in the Materials and methods section. Such a heating step has been used to dissociate the U-A-U-containing ternary transcription complexes formed by calf thymus RNA polymerase II on the poly[d(A-T)] template before the t.l.c. analysis (Vaisius & Wieland, 1982).

Inhibition by α -amanitin under conditions permitting the elongation of RNA

Reaction mixtures were prepared in the presence of wheat-germ RNA polymerase II, poly[d(A-T)] template, U-A primer and either $5 \mu\text{M}$ - $[\alpha\text{-}^{32}\text{P}]\text{UTP} + 5 \mu\text{M}$ -ATP or $5 \mu\text{M}$ - $[\alpha\text{-}^{32}\text{P}]\text{ATP} + 5 \mu\text{M}$ -UTP, such that for both conditions the final specific radioactivity of the labelled nucleotide was the same. In addition, before incubation for 30 min at 35°C , they received various amounts of α -amanitin. Then the reactions were stopped and analysed by high-resolution gel electrophoresis. The autoradio-

Table 1. Best-fit parameters for the inhibition by α -amanitin of U-A-U synthesis catalysed by wheat-germ RNA polymerase II on a poly[d(A-T)] template

U-A-U synthesis was measured in the presence of 21 different concentrations of α -amanitin, ranging from 0 to 10 $\mu\text{g/ml}$. Part of the experimental data is presented in Fig. 2(a). The parameters correspond to eqns. (1) and (2) in the Results section. P_1 and P_2 correspond to the concentration of product U-A-U synthesized in 30 min at 35 °C in the presence of excess α -amanitin and in the absence of α -amanitin respectively. P_3 is the concentration of α -amanitin causing half-maximal inhibition of U-A-U synthesis. Enzyme was at 18 nM. The variance ratio F (Bardsley, 1986) was calculated as indicated in the Materials and methods section. The sum of the residual squares was 0.02436 and 0.08997 with eqns. (1) and (2) respectively.

Eqn. no.	P_1 (nM/30 min)	P_2 (nM/30 min)	P_3 ($\mu\text{g/ml}$)	F
(1)	9.04 ± 1.25	746.5 ± 58.6	0.037 ± 0.004	48
(2)		713.5 ± 72.8	0.052 ± 0.009

Fig. 3. Effect of α -amanitin on poly[r(A-U)] synthesis catalysed by wheat-germ RNA polymerase II

Transcription assay mixtures were prepared as described in the Materials and methods section with poly[d(A-T)] as template, U-A as primer and 18 nM-enzyme. Before incubation for 30 min at 35 °C, they received various quantities of α -amanitin. The Figure shows the radioautographic analysis (150 h exposure at -70 °C) after gel electrophoresis of 5 μl portions (reaction mixtures + stop solution). (a) Lanes 1-7: reactions conducted with 5 μM -[α - ^{32}P]UTP (6.6 Ci/mmol) + 5 μM -ATP. (b) Lanes 1-7: reaction conducted with 5 μM -[α - ^{32}P]ATP (6.6 Ci/mmol) + 5 μM -UTP. Lanes 1, without α -amanitin; lanes 2, + 1.3 μg of α -amanitin/ml; lanes 3, + 2 μg of α -amanitin/ml; lanes 4, + 3 μg of α -amanitin/ml; lanes 5, + 4.4 μg of α -amanitin/ml; lanes 6, + 6.7 μg of α -amanitin/ml; lanes 7, + 10 μg of α -amanitin/ml. For comparison, a reaction assay leading to U-A-U synthesis, such as lane 1 in Fig. 1(a), was analysed in lanes 8.

graph in Fig. 3 clearly shows that the formation of poly[r(A-U)] chains was totally inhibited by α -amanitin. However, only in the case of the use of the [α - ^{32}P]UTP label, a radioactively labelled product co-migrated with U-A-U whose formation was not inhibited by α -amanitin. Hence in the experimental conditions favouring RNA chain elongation as for the single-step addition reaction described in Figs. 1 and 2, a sizeable proportion of the formation of U-A-U escaped inhibition by α -amanitin.

DISCUSSION

The results presented in this paper demonstrate that the pattern of inhibition of wheat-germ RNA polymerase II by α -amanitin exhibits all the seemingly contradictory features that have been reported for the corresponding reactions with the enzymes purified from yeast or mammalian cells. Thus, in the case of the plant enzyme, the formation of a single phosphodiester bond was strongly repressed by α -amanitin, as described for the yeast enzyme (Lescure *et al.*, 1981). A 50 % inhibition of U-A-U synthesis was obtained at 0.04 μg of α -amanitin/ml (Table 1), which compares well with the value of 0.05 $\mu\text{g/ml}$ reported by Jendrisak & Guilfoyle (1978) for half-maximal inhibition of RNA synthesis with the class II RNA polymerases from several higher-plant species.

Vaisius & Wieland (1982) have reported that, even at a concentration of 1 mg of α -amanitin/ml, there was no inhibition of the synthesis of a single phosphodiester bond by calf thymus RNA polymerase II. Such a behaviour is also detected with wheat-germ RNA polymerase II, since the radioautographic analysis in Fig. 1, as well as the determination of P_1 in eqn. (1) (Table 1), give convincing evidence that U-A-U synthesis with the plant enzyme resists to some extent inhibition by excess α -amanitin. We wish to emphasize, however, that the main difference between the cited experiments with the mammalian enzyme and our present results relies, not so much on the extent of formation of a single phosphodiester bond in the presence of excess α -amanitin, but rather on the extent of product formation in the absence of the toxin. Thus the reaction of single-step addition with wheat-germ RNA polymerase II is catalytic, in the sense that U-A-U product concentrations considerably exceed that of the enzyme (see Fig. 2a and the value of P_2 in Table 1). In contrast, with calf thymus RNA polymerase II the amount of U-A-U synthesized on a poly[d(A-T)] template was very low (i.e. nearly stoichiometric with respect to RNA polymerase II in the reactions) and was not significantly different whether the reactions were carried out in the presence or in the absence of α -amanitin (Vaisius & Wieland, 1982). From this comparison, we interpret the data obtained with the plant enzyme to show that the main inhibitory effect of the toxin in the synthesis of U-A-U occurred at a step beyond the formation of the phosphodiester bond. This interpretation is further supported by the observation that U-A-U was the only transcription product formed in the presence of excess α -amanitin under experimental conditions permitting RNA chain elongation (Fig. 3). In this context it is worth noting that α -amanitin at 1 $\mu\text{g/ml}$ does not prevent the formation of transcription complexes with a HeLa-cell extract and adenovirus DNA when the reactions are carried out in the presence of a limited set of ribonucleotide substrates allowing only

the synthesis of a limited number of phosphodiester bonds (Ackerman *et al.*, 1983). Thus in the case of the plant enzyme, as for the mammalian enzyme (Vaisius & Wieland, 1982), the results support the contention that the translocation of the transcription complex along the DNA template is the enzymic process inhibited by α -amanitin.

An interesting implication of the above interpretation would be that with yeast (Lescure *et al.*, 1981) or wheat-germ RNA polymerase II the transcriptional activity in the single-step addition reaction is catalytic (i.e. the trinucleotide product is released by the RNA polymerase) because, following the step of condensation of the nucleotide substrate to the dinucleotide primer, the transcription complexes have undergone some change that could, in view of its sensitivity to α -amanitin, possibly be assigned to a translocation step. (This might correspond to a spontaneous process after completion of nucleotide incorporation or, alternatively, to an abortive event due to the absence from the reaction assay of the 'next' substrate, ATP, dictated by the template sequence.)

Note also that, although our results strongly suggest that the effect of α -amanitin occurs at the step of product release by the enzyme, they do not preclude the possibility that the enzyme-amanitin complex would catalyse the synthesis of a phosphodiester bond at a diminished rate compared with that with the enzyme alone. This would account for the observation that α -amanitin inhibits partly the template-directed process of formation of a phosphodiester bond between a primer nucleotide, covalently bound to the second largest subunit of wheat-germ (Grachev *et al.*, 1986) or yeast (Riva *et al.*, 1987) RNA polymerase II, and a single ribonucleoside triphosphate substrate.

An other relevant aspect revealed by the study of the inhibitory properties of α -amanitin is that the relative probabilities of enzyme translocation with the yeast, wheat-germ and calf thymus RNA polymerases II after formation of the first phosphodiester bond may be important determinants of the rates of single-step addition reaction and productive elongation, and would thereby determine the degree to which the RNA polymerase remains associated in a stable ternary transcription complex with the DNA and the nascent RNA during the first steps of RNA synthesis.

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