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Influence of substrate and template binding on the interaction of α-amanitin with yeast RNA polymerase II: fluorescence spectroscopic analysis

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Fluorescence titration of the tryptophan residues in yeast RNA polymerase II with α-amanitin shows there are two types of binding between the inhibitor and the enzyme. When the stronger binding site was saturated with α-amanitin, the enzyme-inhibitor complex could not be further titrated efficiently with ATP, indicating that probably the inhibitor and the substrate binding domains on the enzyme are overlapping. Supercoiled plasmid DNA bearing alcohol dehydrogenase I promoter of yeast showed binding with purified yeast RNA polymerase II in the absence of substrates. However, when the enzyme-inhibitor complex was titrated with this template, it was found that the complex behaves in a similar way as the enzyme alone towards the template DNA. It suggests that probably the inhibitor and the template binding sites on the enzyme are quite different.

RNA polymerase II; Amanitin, α-; Fluorescence titration; ATP; Promoter, ADC1; (Yeast)

1. INTRODUCTION

The cytotoxin α-amanitin is a bicyclic, octapeptide occurring in high concentrations in the deadly, poisonous mushroom, *Amanita phalloides*. The primary cytopathogenicity of the amanitins is the inhibition of the transcription process catalyzed by RNA polymerase II (ribonucleoside-triphosphate:RNA nucleotide transferase, EC 2.7.7.6) in eukaryotic cells. RNA polymerase II transcribes class II genes giving rise to the precursor mRNA. The half-maximal value for the inhibition of this transcription process by α-amanitin in higher eukaryotes is also known [1].

It is now generally believed that α-amanitin binds RNA polymerase II very tightly with 1:1 stoichiometry [2]. However, the binding was also reported to be reversible [3] in the wheat-germ system. More interestingly, the mode of binding of α-amanitin with RNA polymerase II, in many ways resembles the binding pattern of rifampicin with *Escherichia coli* RNA polymerase. Thus, in direct analogy with the rifampicin action on bacterial transcription, the chemical condensation reaction showed that α-amanitin binds one of the largest subunits of the enzyme [4] and it does not inhibit the formation of the first phosphodiester bond in the RNA product [5], rather it does so for the subsequent ones.

There is a lot of interest currently focussed on the structural analogy of the larger subunits of prokaryotic and eukaryotic RNA polymerases [6,7]. If it is true, then they should have functional similarities also from the point of template, substrate and inhibitor binding. The substrate and the inhibitor rifampicin binding domains in *E. coli* RNA polymerase are now well defined [8,9]. In our continuing effort to develop an in vitro transcription system with yeast [10], we have recently shown the different substrate binding affinities of yeast RNA polymerase II [11]. However, no clear picture has emerged yet on the mode of

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binding of substrate and template DNA with yeast RNA polymerase II in the presence of the inhibitor α-amanitin. Any such knowledge would eventually help in understanding the commonality of the binding sites between substrate or template and α-amanitin with the enzyme as well as the molecular details of the inhibitor action. It has been known for some time now that yeast RNA polymerase II requires a higher concentration of α-amanitin for half-maximal inhibition than that required for the enzyme from animal cell lines [12]. In this report, we have undertaken a detailed analysis of the α-amanitin interaction with the purified yeast RNA polymerase II in the presence of substrate and specific template to gain further insight into the mechanism of action of the inhibitor.

2. MATERIALS AND METHODS

All common chemicals used here were of the highest purity available. Nucleoside triphosphates were purchased from Boehringer and their concentrations were fixed from the known molar extinction coefficient values at the absorption maxima [13]. α-Amanitin was a product of Sigma and [α-^{32}P]UTP was from Amersham or Bhabha Atomic Research Centre, Bombay. A protease negative strain 20B-12 of yeast, *Saccharomyces cerevisiae* was obtained from yeast genetics stock at Berkeley. Growth of yeast cells, purification of the enzyme, its characterization and assay has been reported previously [10,11]. Fluorescence spectroscopic measurements were carried out in a Hitachi 650-10S spectrofluorimeter at 24°C. All the spectra reported here are uncorrected.

The plasmid pAAH5 containing the alcohol dehydrogenase I promoter of yeast [14] was kindly provided by Professor Benjamin Hall, University of Washington, USA, and used as a template here. The DNA-enzyme complex was analyzed by electrophoresing the complex over 0.6% agarose gel in Tris-borate-EDTA (pH 8.3) buffer according to [15].

3. RESULTS

Fig.1 shows the SDS-polyacrylamide gel electrophoretic pattern of yeast RNA polymerase II along with the molecular mass markers. Interestingly we observed that the largest subunit of 220 kDa is not present in our preparation. The enzyme shown in fig.1 had only its proteolysed form, i.e. 185 kDa and 150 kDa larger subunits.

RNA polymerase II prepared in this way showed an intense emission band at 335 nm upon exciting with 280 nm light, mainly due to intrinsic tryptophan residues. In the presence of α-amanitin of varying amounts, the tryptophan fluorescence of

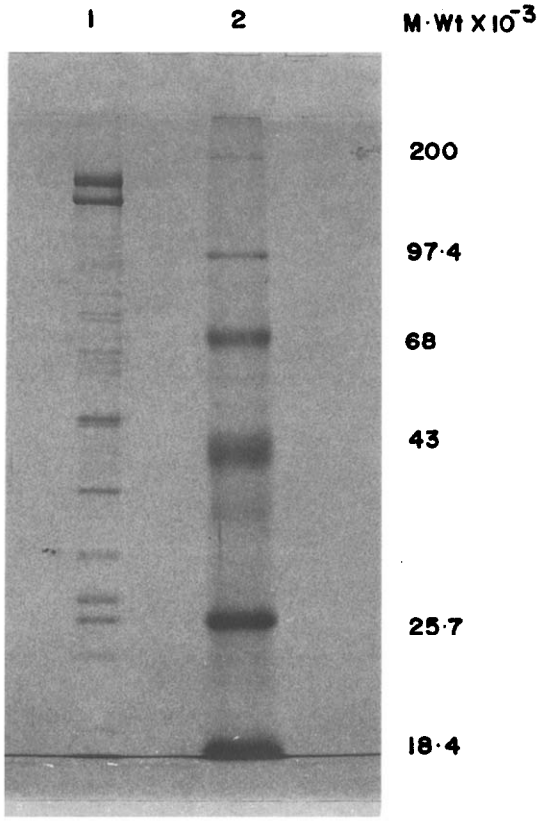

Fig.1. 10% SDS-polyacrylamide gel electrophoretic pattern of yeast RNA polymerase II. Lanes: 1, purified enzyme; 2, molecular mass markers.

the enzyme was quenched as shown in fig.2a. α-Amanitin by itself does not fluoresce; however, its absorption maximum lies around 310 nm where end-absorption overlaps with the tryptophan emission band [3]. Therefore, the quenching of the emission spectrum of yeast RNA polymerase II in the presence of α-amanitin was taken as a measure of the intramolecular energy transfer between the enzyme and the inhibitor as a direct consequence of the complex formation between them.

Due to the presence of finite absorption value of α-amanitin at the excitation wavelength, one has to correct the signal intensity at emission maxima of the titratable tryptophan residues so as to reduce the contribution from inner-filter quenching. This was done by using the equation:

$$\text{intensity}_{\text{correct}} = \text{intensity}_{\text{observed}} \times 10^{(x+y)/2} \quad (1)$$

where x and y are the absorptions of the solution

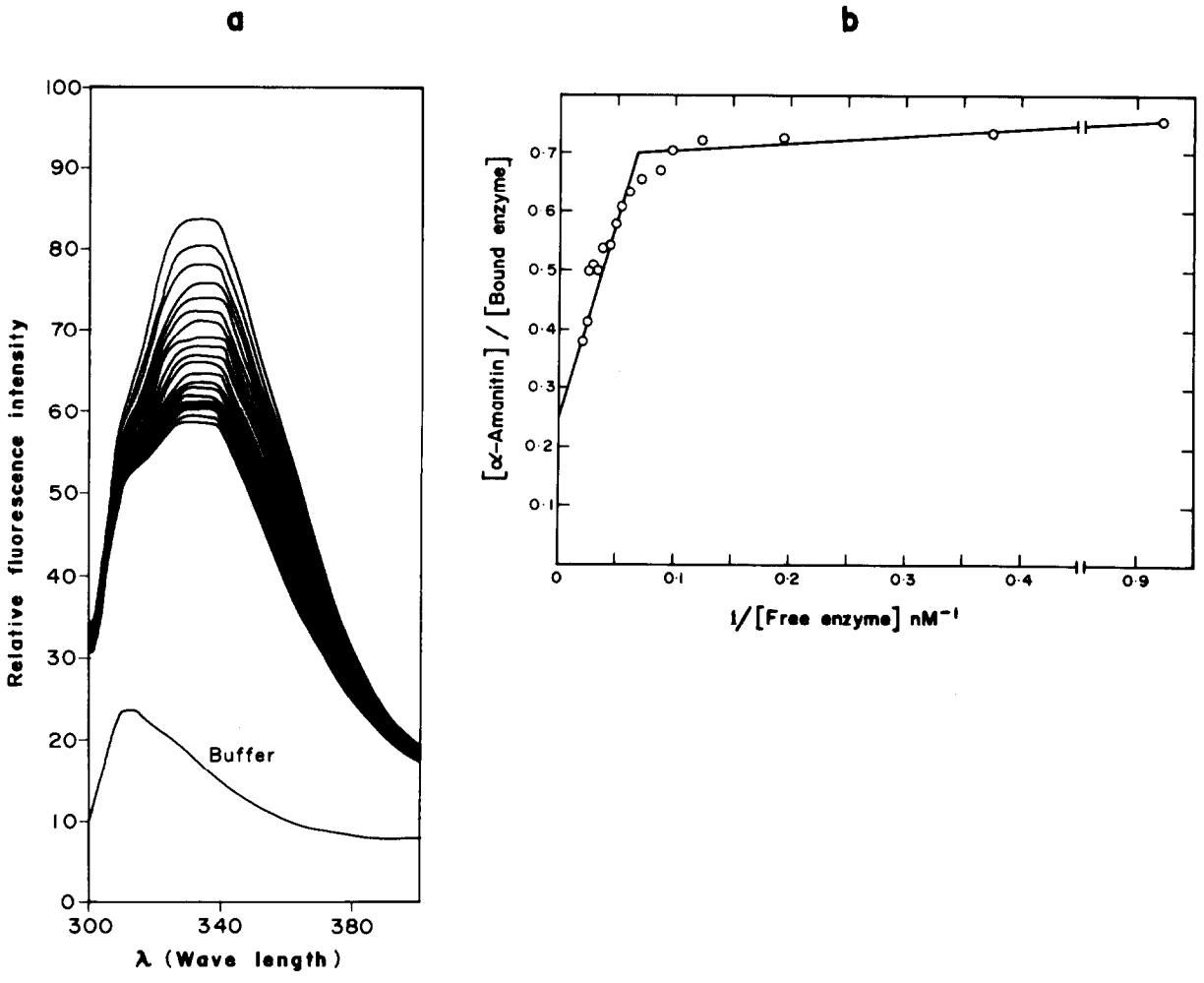

Fig.2. (a) Titration of the intrinsic tryptophan fluorescence of yeast RNA polymerase II (55 nM) with varying concentrations of α-amanitin (0–50 nM). Excitation wavelength was fixed at 280 nm. (b) Binding affinity of α-amanitin with yeast RNA polymerase II. Analysis of the fluorescence titration was carried out according to eqn 3.

at the excitation and emission wavelengths, respectively. Analysis of the fluorescence titration data was carried out in the following way [17]; if there are n number of binding sites (i.e. tryptophan residues) available on the enzyme for α-amanitin, then the mean equilibrium association constant K_a between α-amanitin and the enzyme would be,

$$K_a = \frac{[\text{bound enzyme}]}{[\text{free enzyme}](n[\alpha\text{-amanitin}] - [\text{bound enzyme}])} \quad (2)$$

which on rearranging becomes,

$$\frac{[\alpha\text{-amanitin}]}{[\text{bound enzyme}]} = \frac{1}{nK_a[\text{free enzyme}]} + \frac{1}{n} \quad (3)$$

Computer analysis of the fluorescence titration data was carried out using eqn 3 and is shown in fig.2b.

It was of interest to find out whether the enzyme saturated with α-amanitin would be further titratable with substrate or template. Analysis of such experiments would eventually lead us to understand the overlapping binding domains between them. We reported in [11] on the dissociation constant K_d between ATP or UTP with yeast RNA polymerase II, but no such data exist in the literature for the template-enzyme complex. However, when the plasmid pAAH5 was used as a template, we observed a detectable retardation of

the supercoiled DNA band in the presence of the RNA polymerase II over an agarose gel (fig.3), thereby indicating that the plasmid carrying the promoter region of alcohol dehydrogenase gene (ADC1) was capable of forming a complex with the enzyme in vitro even in the absence of substrates. However, the relaxed DNA was not found to be able to bind the enzyme. This result was not unexpected as it had been reported earlier that supercoiled plasmid bearing strong class II promoter of yeast could form stable complexes with RNA polymerase II [18]. Therefore, we carried out a detailed fluorescence titration study by monitoring the tryptophan emission of the enzyme in the presence of the plasmid (not shown) and

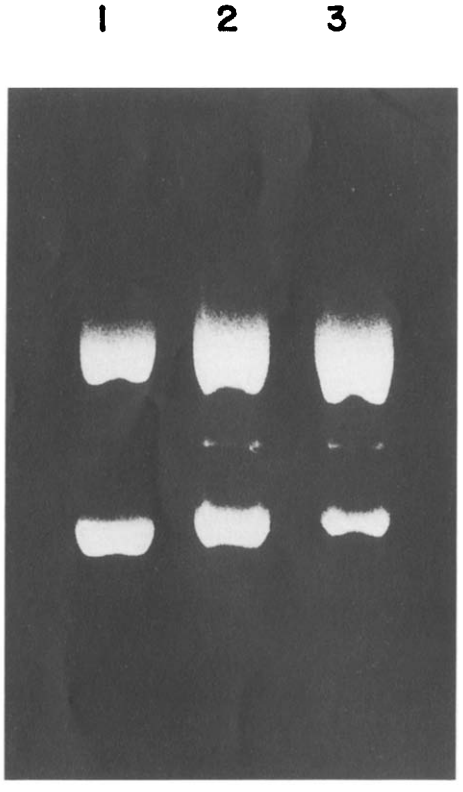

Fig.3. Gel retardation analysis of yeast RNA polymerase II-template DNA complex over a 0.6% agarose gel. Plasmid pAAH5 (3.63 nM) DNA (lane 1) was incubated with 7.26 nM (lane 2) or 14.52 nM (lane 3) enzyme in the presence of 40 mM Tris-HCl (pH 8), 2.5 mM $MnCl_2$, 0.1 mM DTT, 0.1 mM EDTA and 0.2 M KCl at 30°C for 15 min and immediately loaded on the gel after mixing with the dye. Electrophoresis was carried out at 1.5 V/cm until bromophenol blue reached close to the edge of the gel.

Table 1

Binding parameters of supercoiled DNA template (containing alcohol dehydrogenase I promoter) and α-amanitin with yeast RNA polymerase II

Ligand	Type of binding	Binding sites (n)	Equilibrium association constant K_a (M^{-1})
α-Amanitin	1	1.5	5.5×10^9
	2	4.0	3.9×10^7
DNA	1	30	2.6×10^8
	2	50	9.2×10^7

after inner-filter correction (eqn 1) and data analysis (eqn 3) the binding constants between the template DNA and RNA polymerase II were obtained. The K_a values between the enzyme and the inhibitor or the template are shown in table 1.

It can be seen from fig.4a, when tryptophan emission was titrated out with α-amanitin, that ATP titration over the enzyme-inhibitor complex could take place only to a limited extent. On the other hand, fluorescence quenching of the enzyme-inhibitor complex by the template DNA was virtually the same as that in the absence of the inhibitor (fig.4b). More interestingly, substrate titration of the enzyme-inhibitor complex was found to be governed by an isoemittic point (fig.4a).

4. DISCUSSION

Yeast RNA polymerase II has two large subunits: 220 kDa and 150 kDa which constitute the substrate, template and inhibitor binding sites [19]. However, often during isolation of the enzyme, proteolytic cleavage at the C-terminal of the 220 kDa subunit takes place giving rise to a 185 kDa subunit. It was mentioned previously [20] that the control of pH changes, the addition of serine protease inhibitors or the use of a protease negative strain of yeast for enzyme isolation may enable one to prevent the proteolytic cleavage of the 220 kDa subunit. We observed the presence of only a 185 kDa subunit in our RNA polymerase preparation in spite of taking all the above precautions. However, it has recently been reported that the C-terminal domain in this subunit is not essential in vitro for accurate initiation of transcription

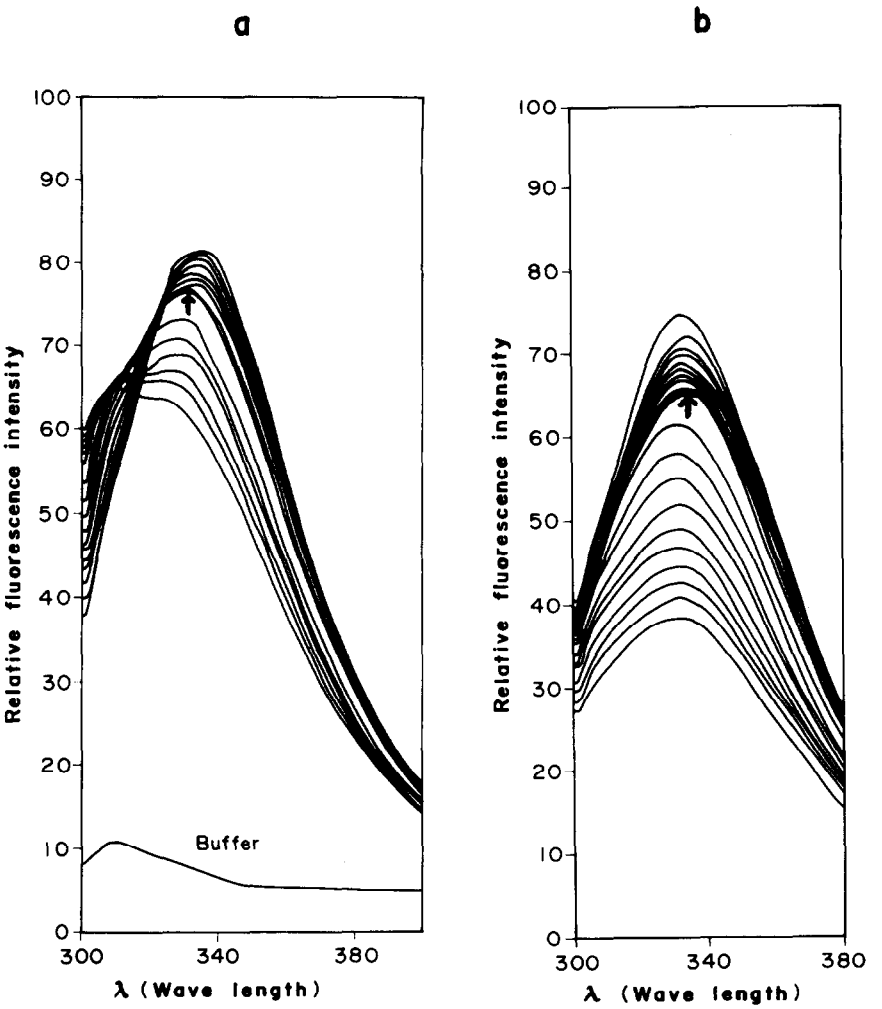

Fig.4. Tryptophan fluorescence titration of yeast RNA polymerase II α-amanitin complex with (a) ATP and (b) supercoiled pAAH5 DNA. Arrow indicates saturation point of the enzyme fluorescence with α-amanitin. [enzyme] = 140 nM; [ATP] = 0–100 μM; [DNA] = 0–2 nM.

[21]. Therefore substrate and template binding properties of our enzyme preparation are not expected to alter very much.

It has already been mentioned before that the binding of the yeast RNA polymerase II with the supercoiled plasmid used here is not surprising, although nothing could be said about the faithful transcription from the alcohol dehydrogenase promoter present in the plasmid DNA. However, we would like to suggest that our data on the binding between this template and the enzyme are more accurate than those that were obtained with a non-specific template.

We have obtained two types of binding between the inhibitor or the template with the enzyme. A large number of binding sites (tryptophan residues) in the template-enzyme complex probably reflects DNA wrapping around the protein over an extended region. Fluorescence titrations of the enzyme-α-amanitin complex with ATP or the template were carried out essentially after saturating the tight binding site of the enzyme with α-amanitin. The concentration of ATP was kept around its K_m as already reported [11]. The presence of the isoemittic point suggests that there is probably one type of complex between enzyme-inhibitor and ATP.

Moreover, the nature of the titration also indicates that α-amanitin and ATP binding sites are probably overlapping. However, template DNA binds distinctly at a different site from that of α-amanitin. Vaisius and Wieland [5] reported earlier that α-amanitin does not interfere with the substrate or template binding to the yeast RNA polymerase II. It is now fairly well established that template binding domains of eukaryotic RNA polymerases are with the two large subunits of the enzyme [22,23]. However, there is a controversy in the literature regarding the binding domain of α-amanitin on the enzyme. Although there was a suggestion [4] that α-amanitin binds the 150 kDa subunit, it conflicts with all the genetic data [19]. Our results presented here do not give an indication of which subunits bind the inhibitor, the template or the substrate, but indicate that the binding sites for the substrate and the inhibitor are close by (therefore probably on the same subunit) and the binding sites of α-amanitin and the template do not overlap (and are therefore probably on the different subunits).

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