Volume 241, number 1,2, 33–37 FEB 06536 December 1988

# Spectroscopic studies on the mode of binding of ATP, UTP and α-amanitin with yeast RNA polymerase II

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Received 23 September 1988

The binding affinity between the substrates ATP and UTP with the purified yeast RNA polymerase II have been studied here in the presence and absence of $Mn^{2+}$. In the absence of template DNA, both ATP and UTP showed tight binding with the enzyme without preference for any specific nucleotide, unlike *Escherichia coli* RNA polymerase. Fluorescence titration of the tryptophan emission of the enzyme by nucleoside triphosphate substrates gave an estimated $K_d$ value around 65 μM in the absence of $Mn^{2+}$ whereas in the presence of $Mn^{2+}$, the $K_d$ was 20 μM. The effect of substrates on the longitudinal relaxation of the HDO proton in enzyme-substrate complex also yielded a similar $K_d$ value.

Fluorescence; NMR; Substrate; Inhibitor; RNA polymerase II; Binding constant

## 1. INTRODUCTION

Although there is general agreement that the control of gene expression resides partially at the level of the activity of RNA polymerases, the exact molecular mechanism responsible for such control is not yet understood. There are various ways such controls can operate and to delineate them selective reconstitution of transcription in vitro is necessary. Thus, the bacterial transcription machinery is well formulated [1] but such is not the case with eukaryotes. Eukaryotic nuclear RNA polymerases I, II and III synthesize ribosomal precursor RNA, pre-mRNA and small stable RNAs respectively [2,3]. These enzymes share the ability to bind DNA, initiate an RNA chain de novo, elongate the nascent RNA, terminate and release the RNA product. The three RNA polymerases are complex enzymes that share certain structural features [3,4]. They are composed of 9–14 polypeptides among which are two very large (135–220 kDa) and many relatively smaller proteins. The two large subunits of RNA polymerase II share some sequence homology and antigenic determinants with the corresponding subunits of RNA polymerase I and III [5]. More interestingly, the two large subunits also share sequence homology with the two large subunits of prokaryotic RNA polymerases [6,7]. Are they also functionally similar to each other? It has been shown previously that the two large subunits of *Escherichia coli* RNA polymerase have different binding sites, one for the substrate and the other for the template [8,9]. Substrate binding to one of the large subunits of *E. coli* RNA polymerase is also known to be purine nucleotide specific, $Mg^{2+}$ and template independent [10]. If the information regarding the nature of interaction of substrate and template with the purified eukaryotic RNA polymerase were available, it would in effect help to reconstitute the active eukaryotic transcription system in vitro. In this communication, we report the simple spectroscopic approach to investigate the nature of the interaction of substrates with purified yeast RNA polymerase II.

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*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/88/$3.50 

## 2. MATERIALS AND METHODS

All common chemicals used were of 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 [11]. $\alpha$-Amanitin was obtained from Sigma. Radiolabelled $\alpha$-[$^{32}$P]UTP was a product of Amersham. A protease negative 20B-12 strain of the yeast, *Saccharomyces cerevisiae*, was obtained from the yeast genetics stock at Berkely. Cells were cultured in an enriched YEPD (0.5% yeast extract, 1% bactopeptone, 0.1% NaCl and 2% dextrose) medium at 30°C and harvested in the late log phase when $A_{600nm}$/ml reached 0.6–0.8. Yeast spheroplasts were prepared according to the method of Cabib [12] using glusulase or zymolase obtained from Sigma. Purification of yeast RNA polymerase II was achieved following the protocol standardised by Valenzuela et al. [13]. Activity of the enzyme over denatured calf thymus DNA was checked at every stage of purification, following a protocol already published by us [14].

Fluorescence measurements were done in a Hitachi 650-10S spectrofluorimeter at 24°C. All the spectra reported here are uncorrected. A Bruker-NMR spectrometer was used for measuring longitudinal relaxation time $T_1$ of water proton at 300 MHz, by the inversion recovery technique using a PD-180°-$\tau$-90° pulse sequence [15]. For this purpose the enzyme which was originally stored in buffer containing 60% glycerol, 20 mM Tris-HCl (pH 8), 0.5 mM EDTA, 10 mM $\beta$-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride was dialyzed for 2 h at 4°C against the same buffer containing 5% glycerol and subsequently precipitated by $(NH_4)_2SO_4$. The pellet was dissolved again in the same 5% glycerol-containing buffer prepared with 98.5% $D_2O$ obtained from Sigma.

## 3. RESULTS

Fig.1 shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis pattern of yeast RNA polymerase II at various stages of purification. It can be seen from the figure that almost all the major subunits of RNA polymerase II are present in this preparation and comparable to the other preparation published elsewhere [16]. The specific activity of the enzyme over denatured calf-thymus DNA was found to be 500 U/mg. The protein showed an intense emission maximum at 335 nm upon excitation at 280 nm, due to tryptophan fluorescence. The intensity of this band was reduced upon the addition of ATP or UTP. However, both ATP and UTP have finite absorption at 280 nm and therefore absorption due to the increasing concentration of triphosphate was corrected to reduce the contribution from inner-filter quenching. Thus, the emission intensity of the titratable tryptophan fluorescence was corrected

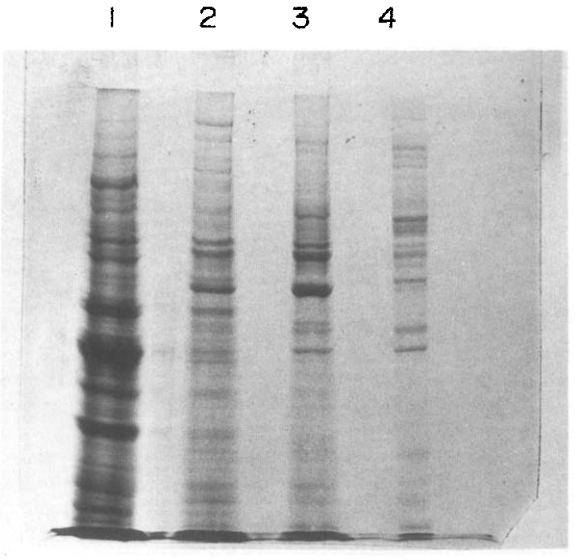


Fig.1. 10% sodium dodecyl sulfate-polyacrylamide gel electrophoretic pattern of yeast RNA polymerase II at various stages of purification. Lane 1, crude extract; lane 2, load of DE-52 column; lane 3, DE-52 eluate or load of phosphocellulose column; lane 4, phosphocellulose eluate.

by using the equation, $\text{Intensity}_{\text{correct}} = \text{Intensity}_{\text{observed}} \times 10^{(x+y)/2}$ [17] where $x$ and $y$ are the absorptions of the solution at the excitation and emission wavelengths respectively. However, the triphosphates do not absorb at 335 nm and therefore the value of $y$ was essentially zero.

Fig.2a shows the fluorescence emission titration of yeast RNA polymerase II with increasing concentration of ATP and UTP in the absence of DNA template. It is clear from the figure that both ATP and UTP bind to the enzyme even in the absence of any meaningful RNA synthesis. Interestingly, it was also observed that in the presence of $Mn^{2+}$, a divalent cation required for the non-specific transcription of RNA polymerase II, both ATP and UTP showed tighter binding with the enzyme (data not shown).

From the different titration curves thus generated, dissociation constant $K_d$ can be approximated from half maximal saturation and table 1 gives all the $K_d$ values obtained in this way.

We have also carried out the measurement of the binding constants between ATP or UTP and yeast RNA polymerase II by titrating the change in water proton relaxation rate [10]. If $1/T_1$ is the

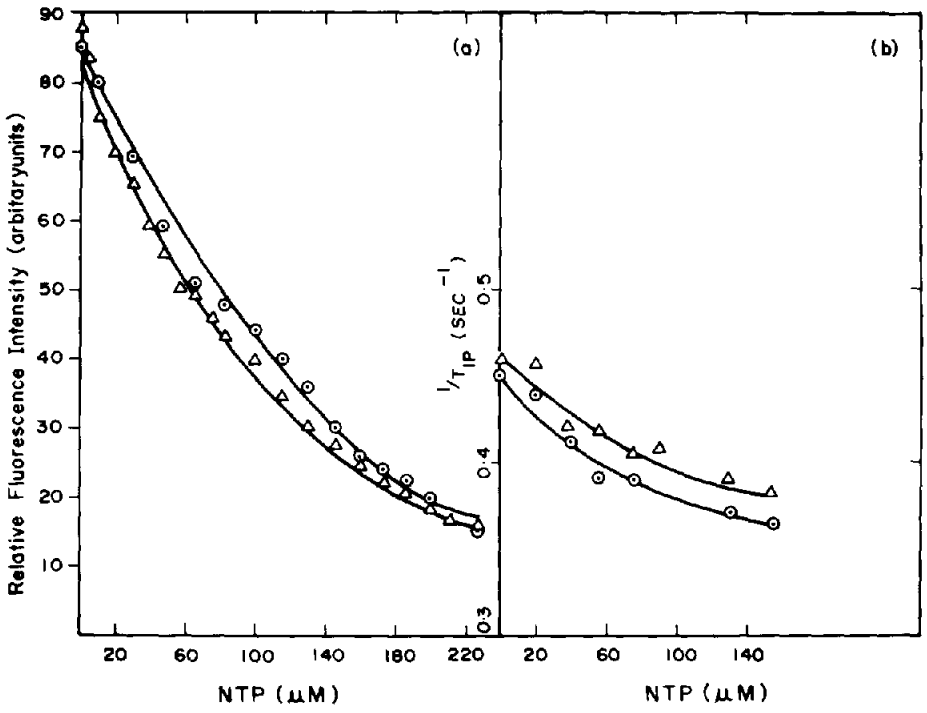


Fig.2. (a) Titration of the tryptophan fluorescence emission of yeast RNA polymerase II with varying concentration of substrates. (○) UTP; (△) ATP. (b) Effect of substrate binding on the longitudinal relaxation of water protons at 300 MHz in deuterated buffer. The concentration of enzyme was $5 \times 10^{-6}$ M.

longitudinal relaxation rate of HDO with a fixed concentration of enzyme then the variation in $1/T_1$ in the presence of different concentration of substrate indicates the accessibility of the substrate to the enzyme under fast exchange limit. However, the presence of paramagnetic $Mn^{2+}$ increases the $1/T_{1(Mn)}$ of HDO remarkably and the difference in $1/T_{1(Mn)} - 1/T_1 = 1/T_{1P}$ with varying concentration of substrate would be a measure of the binding affinity between substrate and enzyme. Fig.2b and table 1 show that the $K_d$ calculated from half-saturation of the HDO titration curve is comparable within error with the fluorescence titration data.

Table 1

Dissociation constant of the substrate-enzyme and inhibitor enzyme complexes for yeast RNA polymerase II

| System[a] | Method | $K_d$ |
|---|---|---|
| (i) ATP-enzyme | fluorescence | 60 μM |
| (ii) UTP-enzyme | fluorescence | 70 μM |
| (iii) ATP-enzyme + $Mn^{2+}$ (2 mM) | fluorescence | 17 μM |
| (iv) UTP-enzyme + $Mn^{2+}$ (2 mM) | fluorescence | 23 μM |
| (v) ATP-enzyme + $Mn^{2+}$ (0.1 mM) | NMR | 50 μM |
| (vi) UTP-enzyme + $Mn^{2+}$ (0.1 mM) | NMR | 45 μM |
| (vii) α-Amanitin-enzyme | fluorescence | 14 nM |

[a] Concentration of the enzyme in the case of fluorescence titration was 0.15 μM and in the case of NMR titration was 5 μM. The concentration of the enzyme was 0.05 μM with α-amanitin

The well-known inhibitor of transcription catalyzed by RNA polymerase II is α-amanitin [18], which also binds the enzyme in the absence of a template. However, it has been reported [13] that yeast RNA polymerase II requires much higher concentration of α-amanitin in comparison to the concentration essential to inhibit 50% of transcription in mammalian cells. Therefore, in order to analyze the binding parameter of α-amanitin with yeast RNA polymerase II, we have also undertaken a fluorescence titration analysis of tryptophan emission with α-amanitin. Table 1 reports here such values.

## 4. DISCUSSION

The exact functions of all the subunits of eukaryotic RNA polymerases are not yet well understood. However, the sequence homology of the larger subunits of different RNA polymerases among each other and also with bacterial RNA polymerases [6,7] raises a further question regarding their functional homology. The large subunit of *E. coli* RNA polymerase, $\beta$-subunit has a specific ATP or GTP binding domain which is template DNA and $Mg^{2+}$ independent [8,9]. This site is known as the initiation site and in most cases the primary transcripts of *E. coli* have ATP or GTP at their 5′ terminus [10]. However, in this communication we show that unlike *E. coli* RNA polymerase, yeast RNA polymerase II does not possess any significantly different purine nucleotide binding site. It should be mentioned here that the processing of the primary transcript in the case of eukaryotes takes place after the elongation of the RNA chain has occurred for a certain length [19] and, so far, no preference for any nucleoside triphosphate at the 5′ terminus of the transcript has been observed [20].

$K_m$ values for the nucleoside triphosphates have been reported for yeast RNA polymerase II [21] under RNA synthesizing conditions. Over denatured calf-thymus DNA this value was found to be within 20–40 $\mu$M. However, we find here that yeast RNA polymerase II also has a template independent substrate binding site. With the analogy of the bacterial system, it appears that this probably is the initiation site of the enzyme. However, both ATP and UTP bind more tightly with the enzyme in the presence of $Mn^{2+}$ than in the absence of it, explaining the basis for the requirement of $Mn^{2+}$ to follow the transcription over specific or nonspecific templates. It can be seen from table 1 that the concentration of $Mn^{2+}$ was much less in the case of NMR titration experiments than what is required for transcription assays (~2 mM). This is particularly so to avoid a large paramagnetic relaxation effect on the water proton. An assumption has been made in the NMR experiments that both the ATP and UTP are fast exchanging with the enzyme in NMR time scale and most of the exogenous $Mn^{2+}$ is enzyme bound.

The simple experimental approach described here also quantitated the binding affinity between $\alpha$-amanitin and yeast RNA polymerase II. Earlier the dissociation constant between $\alpha$-amanitin and RNA polymerase II from CHO cell lines had been estimated to be 0.04 nM [22]. However, in mutant cell lines resistant to $\alpha$-amanitin it was increased to 0.29 nM. In comparison to that, the $K_d$ value of 14 nM reported here indicates very weak binding between yeast RNA polymerase II and $\alpha$-amanitin. This also explains why it requires a very high concentration of $\alpha$-amanitin to see inhibition of transcription by yeast RNA polymerase II compared to the transcription process carried out by other eukaryotic RNA polymerases.

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