Differential Fluorescence and Kinetic Studies on the Template-Binding of RNA Polymerase from Parsley and *Escherichia coli*

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A fluorescence spectroscopy method is described for studying association of RNA polymerase with DNA templates. Using double beam differential fluorescence at excitation and emission wavelengths of 285 and 335 nm, respectively, the new technique discriminates non-specific decrease of fluorescence intensity by addition of DNA from quenching of polymerase fluorescence by protein-nucleic acid interactions. Comparing the results with studies of UMP incorporation into RNA, the K_s-values of template-binding were in good agreement with the values for RNA synthesis, pointing to specific interaction of polymerase and the DNA template measured by the fluorescence method. While *E. coli* enzyme showed higher affinity for templates such as heat-denatured poly [d(A-T)] and poly [d(G-C)] parsley RNA polymerase I accepted such templates with the same affinity as salmon sperm DNA. It is obvious that divalent cations are not necessary for the interaction of both enzymes with single-stranded DNA-templates. UMP incorporation studies suggest that transcription is a cooperative process.

Introduction

The process of RNA synthesis in *E. coli* cells has been subdivided functionally into a sequence of four steps [1]: 1) template-binding, 2) chain initiation, 3) chain elongation and 4) chain termination. Each of these events has a high kinetic complexity and implies the existence of structural domains on the enzyme. Recently we have communicated results obtained by kinetic radiotracer studies which are in good agreement with findings obtained with other techniques which demonstrated that RNA polymerase from *E. coli* has two nucleotide-binding sites. Studies with fluorescence quenching [2] and equilibrium dialysis provided evidence for the presence of a purine nucleotide-binding site on the core enzyme which would correspond to initiation and a second site for binding any of the NTP as a Mg^{2+} chelate which would correspond to the elongation nucleotide site [3]. The binding of purine triphosphates to the initiation site is a slow reaction and is rate limiting for the initiation process followed by the substrate binding to the elongation site which occurs rapidly (for review see [4]). The initiation process does not require divalent cations [3].

The transcriptional machinery of eukaryotes differs in at least one major respect from that of prokaryotes. The nucleus of eukaryotes contains three structurally distinct RNA polymerases with specific roles and properties. During emergence of the eukaryotic line the subunit structures of these enzymes became more complex. Recently we compared RNA polymerase I from higher plants with the enzyme from *E. coli* by use of kinetic radiotracer studies [5]. Thereby eukaryotic RNA polymerase, with its higher complexity of subunit structure, also showed a higher degree of cooperativity of nucleotide binding sites, reflecting a more complex regulation of RNA synthesis in eukaryotes. It is speculated that the eukaryotic RNA polymerase has several binding sites involved in the elongation process which would be of advantage for fitting and proper base pairing between the incoming nucleotides and the template long before the phosphodiester bond is formed during elongation.

Further studies indicated that the true substrate for RNA polymerase I is the complex of NTP and divalent cations; free NTP act as allosteric inhibitors contrary to free divalent cations which promote activity of RNA polymerase [6]. However, besides this cosubstrate effect of divalent cations little is

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known about their further roles in RNA synthesis and in the binding interaction between RNA polymerase and DNA.

In this communication we present a fluorescence spectroscopy method for studying association of RNA polymerase from parsley and *E. coli* with DNA templates, based on a study of fluorescent properties of RNA polymerase and the ability of DNA to quench this fluorescence. The results are compared with radiotracer studies.

Materials and Methods

Freely suspended callus cells of parsley (*Petroselinum crispum*) were cultured according to [7].

RNA polymerase I was purified from parsley according to [8]. RNA polymerase holoenzyme from *Escherichia coli* MRE 600 was purchased from Boehringer (Mannheim).

RNA polymerase activity from *E. coli* was assayed in 0.5 ml reaction mixtures containing 40 mM Tris. HCl (pH 7.9), 75 mM KCl, 8 mM $MgCl_2$, 0.4 mM ATP, CTP and GTP, respectively, 0.04 mM [5′-^{3}H]uridine 5′-triphosphate (4 μCi), 0.1 mM dithiothreitol, 25% (v/v) glycerol, heat-denatured DNA and 0.5 units enzyme protein (1.7 μg, 6.8 nM). The reaction was started by addition of polymerase and incubation was carried out at 33 °C for 30 min. [^{3}H]UTP incorporation into TCA insoluble material was determined; it was linear during incubation period.

RNA polymerase I activity from parsley was assayed in 0.5 ml reaction mixtures containing 50 mM Tris. HCl (pH 8.0), 20 mM $(NH_4)_2SO_4$, 6 mM $MgCl_2$, 0.4 mM ATP, GTP and CTP, respectively, 6 μCi[5′-^{3}H]UTP, 20 mM dithiothreitol, 25% (v/v) glycerol, heat-denatured DNA and 20 μl of the enzyme (10 μg protein, 33.4 nM) so start the reaction. Incubation was carried out at 29 °C for 30 min. [^{3}H]UTP incorporation into TCA insoluble material was measured and was linear during 30 min.

Fluorescence measurements were performed at 23 °C with a Farrand Spectrofluorometer MK 1. The wavelengths of excitation and emission were 285 and 335 nm respectively. A double beam differential fluorescence unit enabled us to carry out fluorometric titrations with RNA polymerase and increasing concentrations of various single-stranded DNA by difference measurements against a reference. The reference contained tryptophan with the same fluorescence intensity as RNA polymerase in the sample cell and represents a non-specific decrease in fluorescence intensity by addition of DNA.

When the relative fluorescence intensity of tryptophan after correction for dilution was plotted against the concentration of DNA represented by optical density at the excitation wavelength, a linear decrease in fluorescence intensity was obtained (not shown). This indicates that the decrease must be the result of the inner filter effect since it is a function of optical density [2]. There is no specific interaction between DNA and tryptophan. With this difference measurement it is therefore possible to discriminate a non-specific decrease in fluorescence intensity by addition of DNA from a quenching of protein fluorescence due to a specific protein-DNA interaction.

In detail the procedure for differential fluorescence measurements was as follows: Sample and reference cells contained 1 ml of buffer with 40 mM Tris-HCl (pH 7.9), 75 mM KCl, 0.1 mM EDTA, 0.1 mM dithiothreitol and $MgCl_2$ as indicated in the figure legends. Fluorescence intensity of *E. coli* RNA polymerase (10 μg of Protein, 20 nM) was measured in the sample cell and adjusted to the same fluorescence intensity by addition of a tryptophan stock solution to the reference cuvet. In the case of parsley RNA polymerase I (10 μg of protein, 16.7 nM) the same procedure was applied. Fluorometric titrations with various heat-denatured DNA templates (in aqua bidest.) were performed by addition of increasing amounts of DNA to the probe as well as the reference cuvet. Thereby the net quenching of polymerase fluorescence can be measured directly without correcting the data. Measured fluorescence spectra of RNA polymerase were not corrected. Because of the difficulties in measuring molar concentrations of the template molecules the dimension $\mu g \cdot ml^{-1}$ was used for the calculations of the K_s- and K_m-values.

Results

RNA polymerase I purified from parsley cells as well as *E. coli* polymerase showed fluorescence spectra with an excitation maximum at 285 nm and an emission maximum at 335 nm. These spectra indicate that the fluorescence of the enzyme proteins is largely due to their tryptophan residues [2]. On

addition of DNA the fluorescence intensity at 335 nm decreases with not detectable shift in the maximum wavelength. As described in Materials and Methods this decrease is in part caused by a specific binding effect while non-specific quenching can be eliminated by difference measurements against a reference containing buffer and tryptophan.

Employing this technique we studied first the binding behaviour of various DNA templates to *E. coli* RNA polymerase. The experiments were carried out both in the absence, and presence, of 8 mM $MgCl_2$ and nucleoside triphosphates (NTP) in order to investigate the influence of these components on protein-DNA interaction. Binding of DNA to RNA polymerase was determined by measurement of net quenching of the polymerase fluorescence as described in Materials and Methods. The data were plotted according to [9] (Fig. 1).

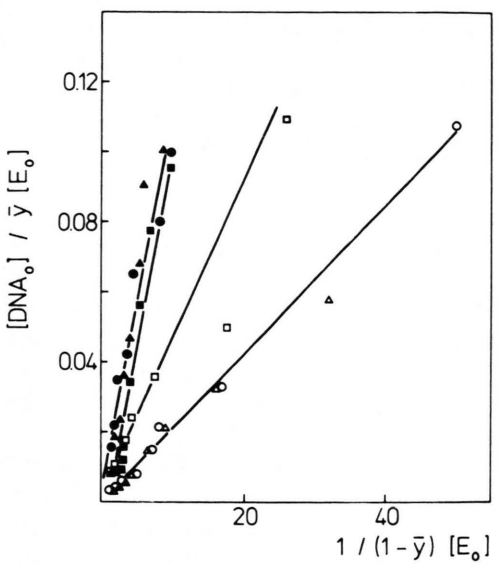

Fig. 1. Fluorometric titrations of *E. coli* RNA polymerase with various single-stranded DNA species. The net quenching of polymerase fluorescence (Δf) obtained by difference measurements against a reference containing tryptophan and the same amounts of DNA as the sample cell was plotted according to Stockell [9]. $\bar{y} = \Delta f / F_\infty$ is the fractional saturation, F_∞ the quenching of polymerase fluorescence when saturated with DNA, [DNA_0] is the total DNA concentration, [E_0] the total polymerase concentration in the assay. The K_s-values were determined from the slopes of the curves. ●—● titration with salmon sperm DNA in the presence of 8 mM $MgCl_2$, ▲—▲ titration with salmon sperm DNA in the absence of $MgCl_2$, ■—■ titration with salmon sperm DNA in the presence of 8 mM $MgCl_2$ and 0.2 mM NTP (0.1 mM each of ATP and GTP), ○—○ titration with poly[d(A-T)] in the presence of 8 mM $MgCl_2$, △—△ titration with poly[d(G-C)] in the presence of 8 mM $MgCl_2$, □—□ titration with poly[d(A-T)] in the presence of 8 mM $MgCl_2$ and 0.1 mM ATP.

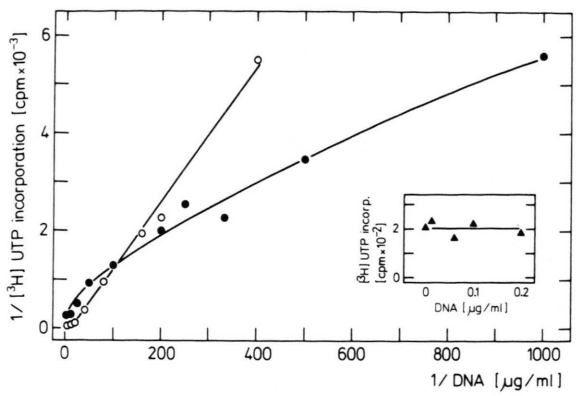

Fig. 2. Kinetic studies of UMP incorporation into RNA with *E. coli* RNA polymerase as a function of increasing concentrations of single-stranded DNA. Data were plotted according to Lineweaver and Burk. K_m-values were calculated by extrapolation of the rate of UMP incorporation obtained at high amounts of DNA (K_m^H). ●—● titration with salmon sperm DNA in the presence of 8 mM $MgCl_2$, ○—○ titration with poly[d(A-T)] in the presence of 8 mM $MgCl_2$. *Inset:* Titration with salmon sperm DNA in the absence of $MgCl_2$.

With denatured salmon sperm DNA as a template and in the presence of 8 mM $MgCl_2$ linear curves were obtained in the Stockell diagram and an apparent dissociation constant of $K_s = 0.0113\ \mu g \cdot ml^{-1}$ could be determined. No difference in the binding behaviour of DNA was observed wether $MgCl_2$ (8 mM) or NTP were present or omitted (Fig. 1). This means that $MgCl_2$ and the purine nucleotides which are necessary for initiation do not influence the association process of polymerase with the DNA template.

In a second set of experiments we studied the interaction between *E. coli* RNA polymerase and DNA using single-stranded poly [d(A-T)] and poly [d(G-C)] as templates. The assays were carried out in the presence of 8 mM $MgCl_2$. As already observed in the case of salmon sperm DNA, linear curves were obtained in the Stockell-plot (Fig. 1), however, clearly lower apparent K_s-values of $0.002\ \mu g \cdot ml^{-1}$ were calculated for both of these templates. When the binding of poly[d(A-T)] was measured in the presence of 0.1 mM ATP the K_s-values became $0.0046\ \mu g \cdot ml^{-1}$ showing an inhibitory influence of ATP on the association process.

To compare the binding step of DNA to RNA polymerase with the synthesis of RNA we measured the UMP incorporation into RNA by *E. coli* RNA

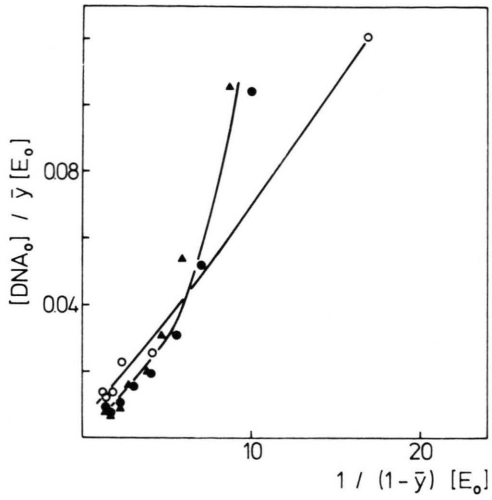

Fig. 3. Fluorometric titrations of parsley RNA polymerase I with various single-stranded DNA species. Data were plotted according to Stockell [9] (see Fig. 1). ○—○ with poly[d(A-T)] in the presence of 8 mM $MgCl_2$, ●—● with salmon sperm DNA in the presence of 8 mM $MgCl_2$, ▲—▲ with salmon sperm DNA in the absence of $MgCl_2$. K_s-values were calculated from the slopes of the linear part of the curves.

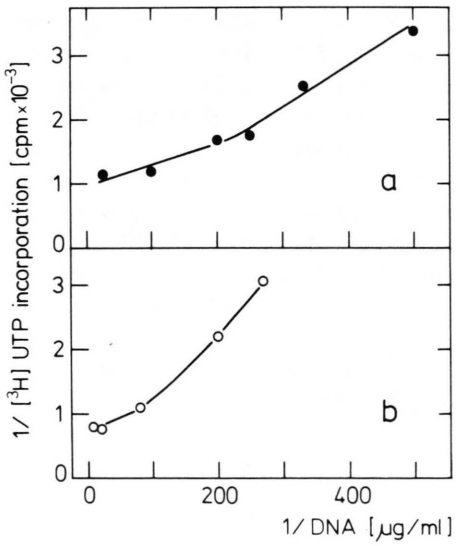

Fig. 4. Kinetic studies of UMP incorporation into RNA by means of parsley RNA polymerase I as a function of increasing concentrations of various single-stranded DNA species. Data were plotted according to Lineweaver and Burk. a) with salmon sperm DNA in the presence of 6 mM $MgCl_2$, b) with poly[d(A-T)] in the presence of 6 mM $MgCl_2$. K_m-values were calculated by extrapolation of the rate of UMP incorporation obtained at high amounts of DNA (K_m^H).

polymerase as a function of DNA concentration with the aid of radiotracer studies. The assays were carried out under approximately the same buffer conditions as used for fluorometric measurements. With denatured salmon sperm DNA as a template and in the presence of 8 mM $MgCl_2$ the Lineweaver-Burk plot revealed a non-linear relationship. By extrapolation of the UMP incorporation rate obtained at high DNA concentrations an apparent K_m^H-value of 0.025 µg · ml^{-1} could be calculated (Fig. 2) which is in the same order of magnitude as the binding constant for DNA.

In the absence of $MgCl_2$ no polymerase activity was observed (inset Fig. 2) since divalent cations are necessary for RNA synthesis by forming chelate complexes with nucleoside triphosphates which are the true substrates for RNA polymerase [6].

With poly[d(A-T)] as a template we obtained non-linear kinetics with an apparent K_m^H-value of 0.08 µg · ml^{-1} (Fig. 2). Compared with the corresponding fluorescence data we got a difference between K_m^H- and K_s-values which can be interpreted by an inhibitor effect of nucleoside triphosphates on the association process (see Fig. 1).

In order to compare the template-binding process of prokaryotic and eukaryotic RNA polymerase we repeated our studies with E. coli polymerase with highly purified RNA polymerase I from a suspension culture of parsley. The data obtained by the fluorescence technique were plotted according to Stockell [9]. Non-linear curves were obtained with denaturated salmon sperm DNA. In the presence as well as in the absence of 8 mM $MgCl_2$ an identical binding behaviour is observed. From the linear part of the curves at high DNA concentrations an apparent dissociation constant of 0.006 µg · ml^{-1} could be determined (Fig. 3). Using poly[d(A-T)] as a template a straight line was found and an apparent K_s-value of 0.006 µg · ml^{-1} could be calculated (Fig. 3). With respect to these K_s-values obtained by the fluorescence technique kinetic studies of UMP incorporation into RNA catalyzed by parsley RNA polymerase I showed similar results (Fig. 4). With increasing concentrations of denatured salmon sperm DNA as a template the Lineweaver-Burk plot revealed a non-linear relationship with an apparent K_m^H-value of 0.004 µg · ml^{-1} (Fig. 4a). When done with the template poly[d(A-T)] a similar non-linear relationship with an apparent K_m^H-value of 0.006 µg · ml^{-1} could be observed (Fig. 4b).

Discussion

Today an ideal method for the analysis of template binding which would allow to measure directly and quantitatively the concentration of any form of binary complex and free RNA polymerase in the assay is not available. Most studies have employed sedimentation analysis or filter binding or have followed complex formation indirectly by measuring RNA chain initiation (for review see [4]). To follow binding of polymerase to DNA these techniques are not practicable because of their low sensitivity, the time consumption and the requirement of large amounts of enzyme. In order to study the interaction of *E. coli* polymerase with nucleotides [2] and with rifampicin [10] the fluorescence of proteins was utilized. Addition of these components to the enzyme solution lead to partial quenching of the protein fluorescence. This effect has been interpreted as a specific interaction of these molecules with one or several tryptophanyl residues in the neighbourhood of the core initiation site on the enzyme [2].

The association of the core enzyme with a template has been demonstrated by fluorescence quenching which is probably due to energy transfer [11]. In this communication the influence of DNA templates on the fluorescence intensity of RNA polymerase holoenzyme from *E. coli* and parsley was utilized to study the binding of the enzyme to DNA.

By comparison of this method with measurements of the incorporation rate of UMP into RNA it was demonstrated that the determination of the quenching of protein fluorescence is an ideal method for the investigation of template binding with the advantage of great inherent sensitivity of fluorescence detection and the need of only low amounts of enzyme.

In a series of experiments with *E. coli* and parsley RNA polymerase and various single-stranded DNA templates K_s-values obtained from fluorescence measurements are approximately the same as the apparent K_m^H-values found by use of kinetic studies of UMP incorporation into RNA. Only in the case of *E. coli* polymerase with poly[d(A-T)] as a template the K_s-value was very much lower than the K_m^H-value determined by the incorporation of UMP. With this template the addition of purine nucleotides resulted in an increase of the K_s-value indicating a change in the association behaviour of polymerase with the template. The data obtained with this fluorescence technique also demonstrated that divalent cations are not necessary for the interaction of prokaryotic as well as eukaryotic RNA polymerase with single-stranded DNA templates since the same dissociation constants were found in the presence and absence of $MgCl_2$.

Comparing the data for the association process between *E. coli* and parsley RNA polymerase with various single-stranded templates the *E. coli* enzyme showed higher affinity for templates such as poly[d(A-T)] and poly[d(G-C)] than for salmon sperm DNA.

In systems with selective transcription of DNA by *E. coli* polymerase binary complexes are formed primarily at promoter regions which are in general AT-rich [12]. The synthetic templates offer *E. coli* polymerase many more binding sites than salmon sperm DNA. Concerning parsley RNA polymerase I denatured salmon sperm DNA as well as poly[d(A-T)] were accepted for association and transcription nearly with the same characteristic. The experiments with UMP incorporation into RNA revealed non-linear curves in the Lineweaver-Burk diagram in all cases, thus indicating that a cooperative process is involved. This cooperativity may be an attribute of the catalytic process since, except in the case of parsley polymerase and salmon sperm DNA, no such effect was observed in the binding experiments. Our results do not allow further conclusions about the nature of this cooperative mechanism and of the apparently negative cooperative effect of salmon sperm DNA on the UMP incorporation mediated by *E. coli* RNA polymerase.

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