FLUORESCENCE MEASUREMENTS ON THE FORMATION OF RNA POLYMERASE–DNA COMPLEXES

P. FAERBER and W. VIZETHUM

Department of Physiological Chemistry, The University of Würzburg Medical School, 8700 Würzburg, GFR

Received 19 November 1975

1. Introduction

Initiation of specific RNA synthesis is considered to start by mutual binding of RNA polymerase to DNA and recognition of a specific structure or sequence within the promoter region. The enzyme is bound to this DNA region at least one order of magnitude more tightly than by non-specific interactions [1]. During our investigations we developed a spectroscopic method which allowed us to follow complex formation of RNA polymerase with different promoters.

It has previously been shown that the fluorescent probe S^6GTP-AEDANS* (I) changes its fluorescence intensity upon interaction with proteins and specific and non-specific protein–nucleotide interactions can be distinguished [2]. We want to present here first results using this system to study the formation of RNA polymerase–DNA complexes.

2. Materials and methods

2.1. *DNA-dependent RNA polymerase (EC 2.7.7.6) holoenzyme*

DNA-dependent RNA polymerase holoenzyme was prepared from *E. coli* MRE 600 cells following the procedure published by Zillig [3] with slight modifications in the first steps. The final purification of RNA polymerase was performed on a DNA affinity column. DNA was coupled covalently to BrCN-activated Sepharose using a β-alanyl spacer. Details of this procedure will be published elsewhere [4]. Core enzyme was prepared from holoenzyme as described by Burgess [5]. The purity of the core- and holoenzyme was better than 98% based on SDS-gel electrophoresis, with a specific activity of 1000 units per mg protein using CT-DNA as template.

2.2. *Assay for RNA synthesis*

The assay was as previously described [6]. The standard incubation mixture contained in a final vol of 100 μl: 4 μmol Tris–HCl (pH 7.9), 400 μmol $MgCl_2$, 100 μmol $MnCl_2$, 1.2 μmol β-mercaptoethanol, template, RNA polymerase and substrates as specified in the legends to figures.

2.3. *Template*

Highly polymerized calf thymus DNA was obtained from Boehringer, Mannheim.

2.4. *Chemicals*

Unlabeled ribonucleosidetriphosphates were obtained from Zellstoff-fabrik Waldhof, Mannheim, GFR, ^{3}H-labeled ribonucleosidetriphosphates from the Radiochemical Centre, Amersham, England. S^6GTP-AEDANS was described previously [2].

2.5. *Spectrophotometric measurements*

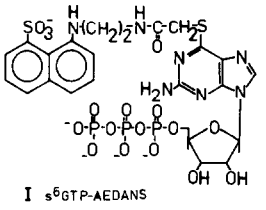
U.v. absorption spectra were recorded with a Hitachi Model 181 or a Cary 15 Model spectrophotometer. Fluorescence measurements were performed with a Schoeffel Spectrofluorometer RRS 1000 with an excitation device in tandem construction. All measurements were carried out in standard buffer: 0.01 M Tris–HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA.

Abbreviations: S^6GTP-AEDANS, 6-thioguanosine-6-*N*-(acetyl-aminoethyl)-8-naphtylamine-1-sulfonic acid-5-triphosphate, CT-DNA, calf thymus DNA.

3. Results

3.1. *Titration of S⁶GTP-AEDANS (I) with RNA polymerase*

When S⁶GTP-AEDANS is mixed with increasing amounts of RNA polymerase-core an increase in fluorescence intensity of the modified nucleotide is observed, concomitant with a blue shift of the emission maximum from 517 to 500 nm (fig.1).



I s⁶GTP-AEDANS

The fluorescence intensity, plotted against the molar ratio of protein to dye, shows a linear increase with a break at a molar ration of about 0.6 (fig.2). The insert in fig.2 demonstrates no further break at high amounts of RNA polymerase-core when mixed with a constant amount of S⁶GTP-AEDANS. This correlation is also observed at other emission wave lengths. About two modified nucleotides (I) are bound to the polymerase in a specific way. The observed stoichiometry may reflect the binding of S⁶GTP-AEDANS to the substrate binding site of the enzyme, but competition experiments have shown that this is not the case. No decrease in fluorescence intensity of the S⁶GTP-AEDANS–RNA polymerase complex was found when high amounts of GTP or any other nucleotide were added. Preincubation of the enzyme with one of the normal

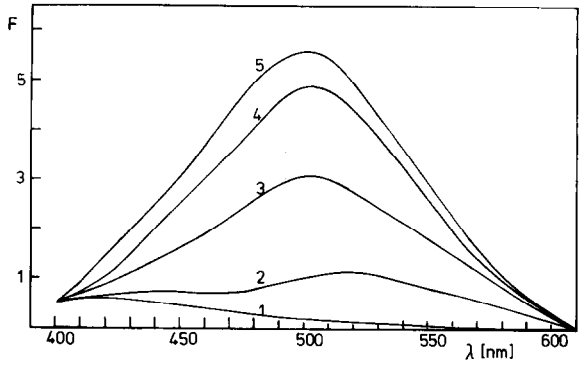


Fig.1. Emission spectra of S⁶GTP-AEDANS during titration with core RNA polymerase in standard buffer. F = relative fluorescence intensity, excitation at 340 nm. Buffer only (1), 6 × 10^{-6} M S⁶GTP-AEDANS (2), molar ratio of protein to dye 0.268 (3), 0.55 (4) and 0.8 (5).

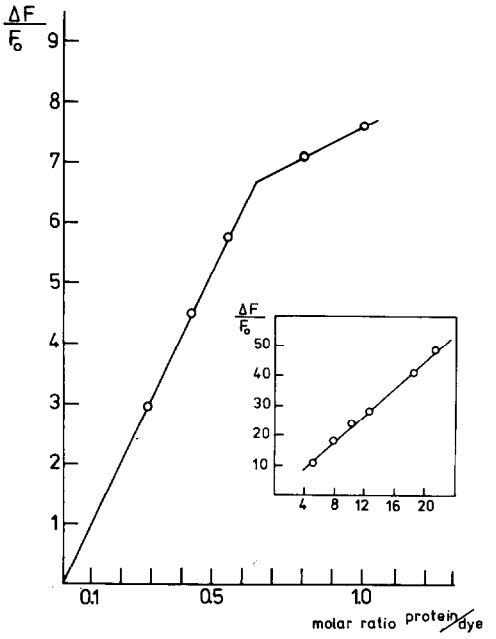


Fig.2. Titration of S⁶GTP-AEDANS (6 × 10^{-6} M) with increasing amounts of core RNA polymerase in standard buffer at 30°C. Excitation at 340 nm, emission recorded at 500 nm. F_0 = starting relative fluorescence intensity, F = difference in fluorescence intensity observed during titration.

Table 1
Complex formation of RNA polymerase with S⁶GTP-AEDANS

Incubation mixture	Additions	F[a]
RNPase, S⁶GTP-AEDANS[b]	–	100
RNPase, S⁶GTP-AEDANS[b]	GTP[c]	100
RNPase, S⁶GTP-AEDANS[b]	GTP,ATP,CTP,UTP[c]	95
RNPase[b], GTP[c]	S⁶GTP-AEDANS	98

[a] F = relative fluorescence intensity

[b] RNPase = RNA polymerase, 2 × 10^{-7} M, S⁶GTP-AEDANS, 4 × 10^{-6} M

[c] Nucleotides, 10^{-3} M each

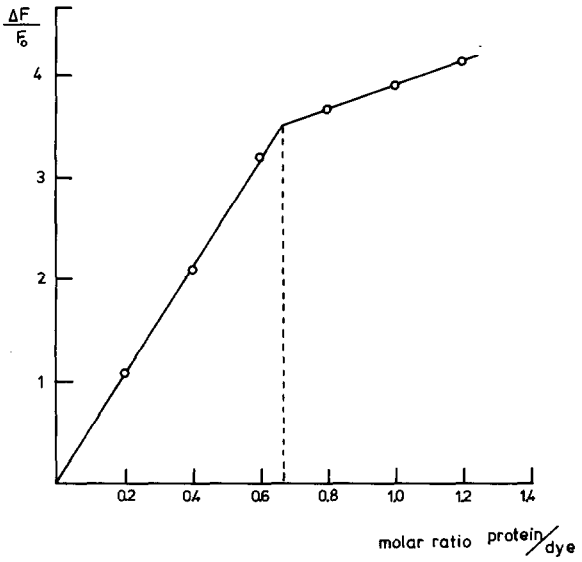


Fig.3. Titration of S^{6}GTP-AEDANS (3.14×10^{-6} M) with increasing amounts of RNA polymerase holoenzyme in standard buffer. For further details see legend to fig.2.

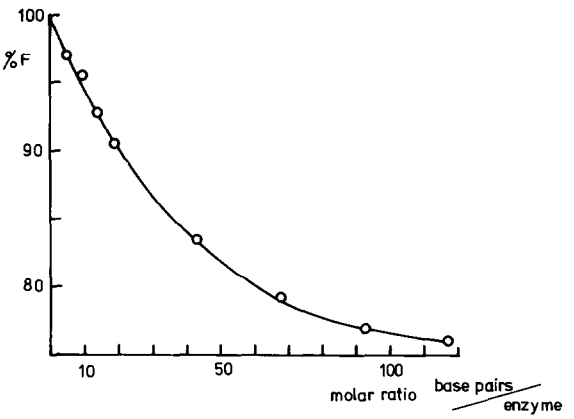


Fig.4. Titration of the polymerase–S^{6}GTP-AEDANS complex (2.06×10^{-7} M) with CT-DNA in standard buffer at 30°C Excitation at 340 nm, emission at 500 nm, F = relative fluorescence intensity; S^{6}GTP-AEDANS alone gives 75% relative fluorescence intensity.

substrates followed by addition of S^{6}GTP-AEDANS shows only a slight effect on the observed fluorescence intensity (table 1).

Similar experiments were performed with RNA polymerase holoenzyme. In fig.3 the titration curve of S^{6}GTP-AEDANS with holoenzyme exhibits a break at nearly the same molar ratio of about 0.65 as found for the core enzyme. At least over a period of 3 h at 30°C the formed RNA polymerase–S^{6}GTP-AEDANS complex is stable under standard assay conditions.

3.2. *Titration of the S^{6}GTP-AEDANS–RNA polymerase complex with DNA*

RNA polymerase and S^{6}GTP-AEDANS were mixed at a molar ratio of 0.04 and the observed fluorescence intensity was taken as 100%. This complex was titrated with increasing amounts of CT-DNA. The fluorescence intensity descreased to a value comparable to that of unbound S^{6}GTP-AEDANS (fig.4). From the plateau value of the titration curve it could be calculated that about 100 DNA base pairs are necessary to remove the bound modified nucleotide from one molecule of RNA polymerase. This is in agreement with the assumption, made by other authors [1], that RNA polymerase covers a DNA region of this magnitude. Nucleotides like GTP or ATP had no influence on the decrease of fluorescence intensity during titration with DNA.

The decrease in fluorescence intensity due to the added DNA is temperature-dependent. No decrease in intensity was observed at 10–15°C in contrast to data obtained at 30°C (table 2). DNA itself shows no detectable interactions with S^{6}GTP-AEDANS (table 2). Preincubation of RNA polymerase with DNA followed by addition of S^{6}GTP-AEDANS results only in fluorescence intensity observed with unbound chromophor.

Since the binding of RNA polymerase with DNA is salt-dependent, similar experiments were performed in the presence of 0.4 M KCl, a salt concentration at which RNA polymerase will not bind to DNA [7]. The complex of RNA polymerase and S^{6}GTP-AEDANS was also effected by high salt concentration. Only 50% of the normally observed increase in fluorescence intensity was found when RNA polymerase and dye were mixed in 0.4 M KCl. No further decrease in fluorescence intensity occured when this complex was titrated with DNA. The intrinsic tryptophan fluorescence of RNA polymerase with an emission maximum at 335 nm was not influenced by S^{6}GTP-AEDANS, although this compound is excitated at 340 nm.

Table 2
Effect of DNA on the RNA polymerase–S^{6}GTP-AEDANS complex

Incubation mixture	Additions	t	F[a]
RNPase, S^{6}GTP-AEDANS[b]	–	30°C	100
RNPase, S^{6}GTP-AEDANS[b]	CT-DNA[c]	30°C	75
RNPase, S^{6}GTP-AEDANS[b]	CT-DNA[c], GTP[d]	30°C	73
RNPase, S^{6}GTP-AEDANS[b]	CT-DNA[c]	15°C	98
RNPase, S^{6}GTP-AEDANS[b]	CT-DNA[c], GTP[d]	15°C	99
RNPase[b], CT-DNA[c]	S^{6}GTP-AEDANS[b]	30°C	75
S^{6}GTP-AEDANS[b]		30°C	74
S^{6}GTP-AEDANS[b]	CT-DNA[c]	30°C	75

[a] F = relative fluorescence intensity

[b] RNPase = RNA polymerase, 2×10^{-7} M, S^{6}GTP-AEDANS, 5×10^{-6} M

[c] CT-DNA, 2.3×10^{-5} M, expressed as base pairs

[d] GTP, 10^{-3} M

3.3. *Transcription of CT-DNA in the presence of S^6 GTP-AEDANS*

The transcription of DNA under standard conditions (see Materials and Methods) is not influenced by S^6 GTP-AEDANS in the concentration range used for the fluorescence measurements described above (table 3). Same results were obtained if the dye was added during transcription or if the enzyme was pre-incubated with S^{6}GTP-AEDANS and RNA synthesis started by addition of nucleotides and DNA. Only when the molar ratio of RNA polymerase to dye was raised up to 0.0075, a slight decrease in activity was found.

4. Discussion

The formation of complexes between RNA polymerase and ribo- or deoxyribonucleotides has been studied with a variety of different techniques [8,9] but no direct spectroscopic method has been reported so far.

In the present work we show that RNA polymerase complexed with the modified nucleotide, S^{6}GTP-AEDANS, loose its fluorescence label if DNA–enzyme interactions take place as measured by a decrease in fluorescence intensity.

From the experimental results evidence could be

Table 3
Transcription of CT-DNA with RNA polymerase in the presence of S^{6}GTP-AEDANS

Incubation mixture	S^{6}GTP-AEDANS (μmoles)	Molar ratio enzyme/dye	Incorporation nmoles ^{3}H GMP/ 10 min
RNPase, mix[a]	3.75×10^{-4}	0.04	7.6
RNPase, mix[a]	2.0×10^{-3}	0.0075	5.2
RNPase, mix[a]			7.5

[a] RNPase = RNA polymerase, 1.5×10^{-5} μmol, GTP,ATP,CTP,UTP, 10^{-3} M each, standard incubation conditions using 10 μg CT-DNA as template

derived that S^6GTP-AEDANS is not bound at or near the substrate binding site of the enzyme, since the presence of high amounts of nucleoside-5′-triphosphates did not influence the binding of the dye to the enzyme. This is also in agreement with the finding that the tryptophan fluorescence of the enzyme is not quenched, which would be expected if the fluorescent label is bound at the substrate binding site [10].

These spectroscopic results were confirmed by transcription experiments in the presence of S^6GTP-AEDANS. Only with high amounts of the modified nucleotide a decrease in nucleotide incorporation was detected.

The increase in fluorescence intensity observed during titration of the dye with polymerase is almost completely reversible when DNA is added to the preformed complex. RNA polymerase–DNA complexes show no further complex formation with S^6GTP-AEDANS. From these results it can be assumed that the modified nucleotide is bound at or near the DNA binding site of the enzyme, since DNA binding is a prerequisite for removing the label from the enzyme. This assumption was further corroborated by experiments under conditions which do not allow RNA polymerase–DNA complex formation, e.g. high ionic strength or low temperature. In these experiments the fluorescence intensity of the enzyme–dye complex was not affected by added DNA.

The abnormal binding behaviour of S^6GTP-AEDANS has to be ascribed to the modification of the purine base with a bulky substituent and is therefore not recognized as a substrate by the enzyme.

The system presented provides a convenient method to follow directly DNA complex formation with native RNA polymerase, in contrast to the methods developed up to now. Stopped flow measurements are now in progress to study the kinetics of RNA polymerase-promoter interactions.

Acknowledgements

We deeply appreciate the pertinent comments and suggestions of Professor Dr D. Palm; our thanks are due to Dr K. Schnackerz for his comments and reading the manuscript. This work was supported by research grants from the Deutsche Forschungsgemeinschaft.

References

[1] Sethi, V. S. (1971) Progr. Biophys. Mol. Biol. 23, 67.
[2] Faerber, P. and Vizethum, W., in the press.
[3] Zillig, W., Zechel, K. and Halbwachs, H.-J. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 221.
[4] Faerber, P. and Vizethum, W. in preparation.
[5] Burgess, R. R. (1969) J. Biol. Chem. 244, 6160.
[6] Faerber, P. (1973) Biochem. Biophys. Res. Commun. 55, 873.
[7] Jones, O. W. and Berg, P. (1966) J. Mol. Biol. 22, 199.
[8] Richardson, J. P. (1966) J. Mol. Biol. 21, 115.
[9] Hinkle, D. C. and Chamberlin, M. J. (1972) 70, 157.
[10] Wu, C. W. and Goldthwait, D. A. (1969) Biochemistry 8, 4450.