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Conformational changes of *E. coli* RNA polymerase during transcription initiation

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Abstract

Escherichia coli RNA polymerase-promoter complex undergoes a multistep process to initiate transcription. We have employed fluorescence spectroscopic approaches to detect the conformational states of the enzyme during this multistep process. A fluorescence assay based on the measurement of fluorescence of free and promoter-bound enzyme as a function of temperature within the range of 4 to 37°C showed that, starting with initial 'closed complex', there are conformationally two distinct intermediate states of the polymerase till it attains the final form required for transcription initiation. The equilibrium from closed complex (RP_c) to open complex (RP_o) consists of at least the following two intermediate complexes:

$$RP_c \overset{4\text{–}13^\circ C}{\rightleftarrows} RP_{i1} \overset{13\text{–}19^\circ C}{\rightleftarrows} RP_{i2} \overset{19\text{–}24^\circ C}{\rightleftarrows} RP_o$$

Higher order structure of RNAP in each of these complexes was probed by means of measurement of accessibilities of the tryptophan fluorophores to the acrylamide. In the next part of the study, TbGTP, a fluorescent substrate, has been used to probe the state of active site in the enzyme for the complexes RP_c, RP_{i1}, RP_{i2} and RP_o, respectively. From the comparison of changes in the parameters such as, fluorescence polarization anisotropy of TbGTP and its accessibility to the neutral quencher, acrylamide, in free and promoter-bound enzyme, we have further substantiated the first part of our results. Together these results suggest that formations of RP_c and RP_{i1} do not involve radical conformational changes in the enzyme, while the enzyme undergoes major change in conformation in the steps $RP_{i1} \rightarrow RP_{i2}$ and $RP_{i2} \rightarrow RP_o$. The strong tryptophan promoter cloned in plasmid pDR720 was chosen as a model promoter in these studies.

Keywords: RNA polymerase; Fluorescence; Acrylamide quenching; TbGTP complex

1. Introduction

Escherichia coli RNA polymerase (RNAP) initiates transcription from its cognate promoter through a sequential multistep process [1–7]:

$$\text{RNAP(R)} + \text{Promoter(P)} \underset{\text{(bind)}}{\rightleftarrows} RP_c \underset{\text{(nucleate)}}{\rightleftarrows} RP_i \underset{\text{(melt)}}{\rightleftarrows} RP_o$$

where, RP_c, RP_i and RP_o are closed, intermediate and open complexes, respectively. Earlier footprinting and gel retardation studies [8–13] revealed that

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these intermediate complexes could be trapped at temperatures below 21°C. There are few studies addressing the conformation of RNAP in these intermediates [14–19]. Low resolution studies with neutron and X-ray scattering provided topological information about RNAP in free and promoter-bound form at 37°C [14,16,20]. In a recent report [21] subunit–subunit interaction of RNAP in its complex with lacUV5 promoter was analyzed using a cross-linking agent like formaldehyde, during transcription initiation.

In our laboratory, we are attempting to understand the conformational states of RNAP during the above steps of transcription initiation by means of a fluorescence assay based on the change in internal fluorescence of RNAP due to the formation of the complex with promoter. We have addressed the following two questions. What are the number of steps from $RP_c \rightarrow RP_o$ in terms of conformational changes of the enzyme? What are the possible conformational alterations from the native enzyme which occur during these steps? Results related to the first part of the problem have been reported recently [22]. After initial characterization of the steps and a preliminary idea about the gross conformational changes of RNAP, the second part of our report describes experiments to identify the changes in active site conformation of the enzyme in these steps. A fluorescent substrate, TbGTP complex, has been used as the probe for the purpose.

Interaction of RNAP with the *trp* promoter leads to quenching of tryptophan fluorescence. Such quenching is a common feature at all temperatures from 4 to 37°C. We have monitored gradual changes in the extent of quenching as a function of temperature. From the temperature dependence of the quenching, we have demonstrated three different equilibria during the whole process [22]. We have also identified four temperatures, at each of which, a near homogeneous population of each one of RP_c, RP_{i1}, RP_{i2} and RP_o is obtained. The conformation of the enzyme in each of these complexes was probed from the quenching pattern of tryptophan residues to a neutral quencher, acrylamide [23]. The quenching patterns were analyzed to compare the relative accessibilities of tryptophan residues in each intermediate, because any change in accessibility originates from a conformational transition of the enzyme. In order to investigate the changes in active site conformation of this enzyme during the formation of RP_o, we have used a fluorescent substrate, namely TbGTP complex. Tb(III) ion, otherwise nonfluorescent, shows strong luminescence at 488 nm and 545 nm (λ_{ex} = 295 nm) when it forms complexes with guanosine nucleotides [24]. TbGTP binds to RNAP with high affinity ($K_d = 0.2$ μM). It is incorporated into mRNA message [24]. Therefore this probe is a suitable reporter for the changes occurring at the active site during initiation. Fluorescence polarization anisotropy (FPA) and accessibility of the probe to the solvent, monitored from its extent of quenching with acrylamide are the two experimental parameters.

A strong *trp* promoter (cloned in the plasmid pDR720) [25] with high affinity for RNAP, was chosen as the model promoter for the following reasons. According to different prediction methods of promoter strength based on its sequence analysis [26,27] and functional assays of the *trp* promoter [25], it is established that *trp* is a strong promoter like T7A1, tac etc. Equilibrium constants for these promoters are found to be greater than 10^9 M^{-1} from in vitro and in vivo studies [6,7,28]. Therefore under present experimental conditions ([pDR720] = 12 nM, see Materials and methods), there will be a homogeneous population of the *trp* promoter–RNAP holoenzyme complex in spite of the presence of two other relatively weaker promoter sites in the plasmid. Furthermore forward rate constant of closed complex formation is ca. 10^{10} M^{-1} s^{-1}. These values are 1000-fold higher than those observed for weak promoters. Initial low temperature (5°C) used in the present study would not lead to dissociation of the closed complex, because cryoelectron microscopic studies at 0°C [29] have shown the formation of a stable closed complex between holoenzyme and strong promoter, T7A1. On the other hand, low temperature will lead to dissociation of the holoenzyme from a weak promoter binding site [9,11].

Circular DNA is preferred to a linear restriction fragment because it mimics in vivo conditions where higher order structure of circular DNA might play a role in promoter recognition. Use of circular DNA also eliminates any strong non-specific binding at the ends of linear DNA, which is about 600-fold stronger than the interior non-specific sites [6].

2. Materials and methods

E. coli RNA polymerase (Lot no: CG0916103) of ultrapure quality was purchased from Pharmacia-LKB Biotechnology Ltd. $TbCl_3$ was purchased from Aldrich Chemical Company. Plasmid pDR720 [25] was isolated from *E. coli* JM101 cell and purified from low melting agarose gel according to the standard procedure [30]. The strong *trp* promoter is cloned upstream of the galK gene in the expression vector pDR720. This is derived from the plasmid pBR322. Purity of the plasmid DNA was checked from agarose gel electrophoresis and it was found to contain less than 10% of nicked form. It was also free from fluorescent impurities. RNAP was FPLC pure and saturated with σ-factor; its purity was further checked in SDS-PAGE followed by silver staining. The specific activity of the enzyme was 950 units/mg (4900 units/ml) in terms of incorporation of 1 nmole of AMP into acid soluble product using poly(dA) · poly(dT) as template in 10 min at 37°C. Experiments with $TbCl_3$ were done in the following buffer: 20 mM Tris–HCl, pH 7.5, 100 mM KCl (buffer T). Tb(III) tends to precipitate out as $Tb(OH)_3$ at higher pH. Thus the pH of the buffer was kept 7.5; this is optimal with respect to the enzyme activity and the stability of Tb(III) in solution. For other experiments, buffer with the following composition was used: 50 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, and 1 mM DTT (buffer A). Buffers were prepared with Milli Q (Millipore Corporation, USA) water. Other reagents were of analytical grade.

Fluorescence spectra of the enzyme were recorded with Hitachi 4010 Spectrofluorimeter in a CAT mode of multiple scans. The excitation wavelength was 295 nm with excitation and emission slit widths of 5 nm and 10 nm, respectively. Appropriate substraction of any contribution from buffer was done. Photodegradation was tested and found to be absent for the enzyme and TbGTP complex alone and in presence of promoter. Correction due to inner filter effect was not incorporated because absorbance of the samples did not exceed 0.04.

2.1. Fluorimetric assay of conformational changes for RNA polymerase

In these studies, holoenzyme (10 units) was incubated with supercoiled plasmid DNA (12 nM) at 4°C and the temperature was increased at intervals of 1 to 2 degrees up to 37°C. At each temperature, the system was allowed to equilibrate for 15 min. The fluorescence spectrum was recorded after this time. Temperature dependence of the fluorescence spectra for free enzyme was also carried out under identical conditions. Relative emission intensity (F_{free}/F_{bound}, where F_{free} and F_{bound} denote emission intensities of RNAP, free and in presence of pDR720, respectively, at 340 nm) of RNAP in buffer A was monitored as a function of temperature from 4 to 37°C.

2.2. TbGTP complex as active site probe

At each temperature the TbGTP complex was made by mixing the both components according to the available method [24]. RNAP was then added and sufficient time was allowed for its binding to the TbGTP complex. This was repeated at four different temperatures. Choice of temperature followed the result from the previous set of experiments. A saturating amount of plasmid pDR720 was added to the preformed TbGTP–RNAP complex at these specified temperatures. Fluorescence polarization anisotropy (FPA) of the TbGTP complex (λ_{ex} = 295 nm, λ_{em} = 545 nm) under different conditions was measured at these temperatures. Their quenching patterns to acrylamide were also evaluated under identical conditions.

2.3. Analysis of FPA

It was measured using the following expression: $\langle r \rangle = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where I denotes the intensity and the subscripts refer to vertical or horizontal positioning of excitation and emission polarizers respectively [31]. $G = (I_{HH}/I_{HV})$ was used to correct the polarization effects in the emission monochromator and detector.

2.4. Analysis of quenching patterns

The relevant data were analyzed according to the Stern–Volmer method using the following equation [32]:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are fluorescence intensities of the

enzyme in absence and presence of the quencher, respectively. K_{SV} and $[Q]$ denote quenching constant and input concentration of quencher, respectively. Initial slope of the line for F_0/F against $[Q]$-plot gives K_{SV} in case the experimental points show a deviation from linearity. Fraction of accessible (f_e) tryptophan residues in RNAP under different conditions was evaluated from the following equation [32,33]:

$$F_0/(F_0 - F) = 1/(K_{SV} f_e[Q]) + 1/f_e \qquad (2)$$

3. Results

3.1. Change in intrinsic fluorescence of free and promoter-bound RNAP with temperature

Fig. 1 shows the ratio of fluorescence intensity of free and bound RNAP as a function of temperature in the range 4 to 37°C. The ratio was taken to normalize the effect of temperature on fluorescence of the free enzyme. The resulting plot could be described as the sum of three hyperbolic curves or

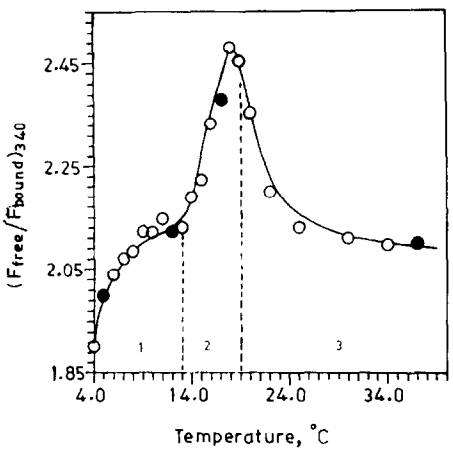

Fig. 1. Relative emission intensity (F_{free}/F_{bound}, where F_{free} and F_{bound} denote emission intensities of free RNAP (10 units) and in presence of 12 nM of pDR720, respectively, at 340 nm) of RNAP in buffer A as a function of temperature (-O-O-). Temperature ranges for different equilibria are demarcated by broken lines and the equilibria 1, 2 and 3 (as described in results) are indicated by numerals 1, 2 and 3. Temperatures corresponding to closed circles in the graph were chosen for probing the conformations of RNAP alone and in complexes.

part thereof. Similar results were obtained with two different batches of plasmid DNA. The multiphasic nature could originate from transition of one type of complex to another with a different fluorescence quantum yield. It could be ascribed to the following equilibria involving four complexes starting with the closed complex at 4°C:

$$RP_c \overset{4-13°C}{\rightleftarrows} RP_{i1} \quad \text{equilibrium 1}$$

$$RP_{i1} \overset{13-19°C}{\rightleftarrows} RP_{i2} \quad \text{equilibrium 2}$$

$$RP_{i2} \overset{19-24°C}{\rightleftarrows} RP_o \quad \text{equilibrium 3}$$

These equilibria are clearly marked in Fig. 1. The final complex at 24°C is a stable form; therefore, it does not show any further significant change in fluorescence ratio as a function of temperature. This trend agrees with earlier reports of formation of different intermediates culminating in open complex from closed complex during transcription initiation [5,8,13,14]. We picked up characteristic temperatures corresponding to the formation of each complex. 5°C is an obvious choice because RNAP remains a closed complex at this temperature. Other temperatures were 12, 17 and 37°C. These temperatures (marked in Fig. 1) were chosen to get a nearly homogeneous population of each complex.

3.2. Characterization of RNAP–promoter complexes in terms of conformations of RNAP

Four different temperatures, namely 5, 12, 17 and 37°C, were chosen to probe the accessibilities of tryptophan residues in RNAP in free and bound form. A change in 3° or 4° structures of the enzyme would result in an alteration of the accessibility of the tryptophan residues. Thus quenching studies provide a means to characterize RNAP–promoter complexes in terms of conformations of RNAP. Table 1 summarizes the relevant parameters from quenching studies. It suggests that K_{SV} values for tryptophan residues in bound RNAP do not follow any general trend. This contrasts tryptophan residues of free enzyme, where we notice an increase in quenching constant with temperature as a typical feature for dynamic quenching. The apparently anomalous

Table 1
Quenching constants (K_{SV}, M^{-1}) of tryptophan residues for *E. coli* RNAP under various conditions in buffer A

Temperature (°C)	RNAP alone	RNAP in presence of *trp* promoter	K_{SV}(bound)/ K_{SV}(free) [a]
5	20.3	27.4	1.35
12	28.3	25.7	0.90
17	47.8	16.6	0.35
37	66.75	38.3	0.60

[a] Terms in parentheses denote the state for RNAP with respect to the promoter.

change in quenching constants for tryptophan residues in bound RNAP might originate from different environments of the fluorophore in the complex at four temperatures. This is confirmed from the measurement of the accessibilities of the fluorophores by means of modified S–V plots (Eq. 2). The results are summarized graphically in Fig. 2. The figure clearly indicates that the RNAP–promoter complex behaves differently in terms of accessibilities of *trp* residues of the enzyme at four temperatures. These experiments help us to suggest that there is a temperature induced transition in the 3° or 4° structure of RNAP when it is bound to the promoter during transcription initiation.

3.3. The TbGTP complex as a probe for active site conformation of RNAP during transcription initiation

Results presented above indicate that the conformational state of the enzyme in RNAP–promoter complex are different at four temperatures, where RP_c, RP_{i1}, RP_{12} and RP_o are formed. As a consequence of these results we have employed the TbGTP complex, as a probe for the active site of the enzyme in these complexes.

At each of the above temperatures, complex formations of TbGTP with RNAP and TbGTP–RNAP with promoter were confirmed from quenching of TbGTP emission signal at 545 nm. The quenching of fluorescence of TbGTP complex in presence of RNAP is in accordance with an earlier report [24]. Representative spectra are shown in Fig. 3. Stability of TbGTP complex ($K_d = 0.2$ μM) is greater than

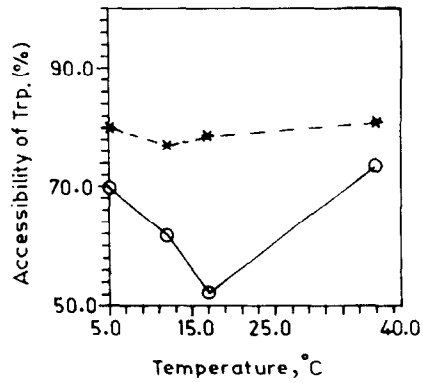

Fig. 2. Changes in accessibility of tryptophan residues of RNAP (10 units), alone (–★–★–) and in presence of 12 nM pDR720 (–○–○–) as a function of temperature between 4°C and 37°C in buffer A.

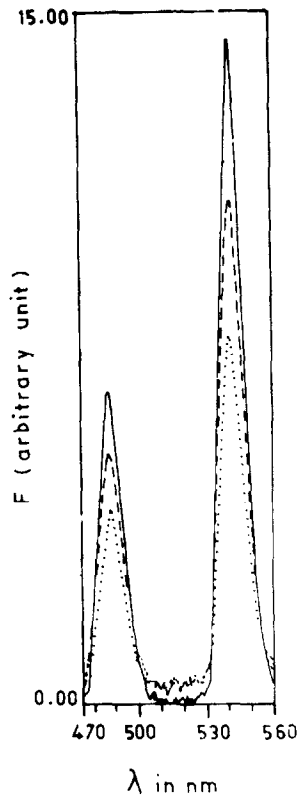

Fig. 3. Fluorescence emission spectra of TbGTP ([Tb(III)] = 77 μM, and [GTP] = 12 μM) alone (————) and in presence of (i) 10 units of RNAP [– – –], (ii) 10 units of RNAP plus 12 nM of pDR720 (....) in buffer T at 17°C. Excitation wavelength was 295 nm.

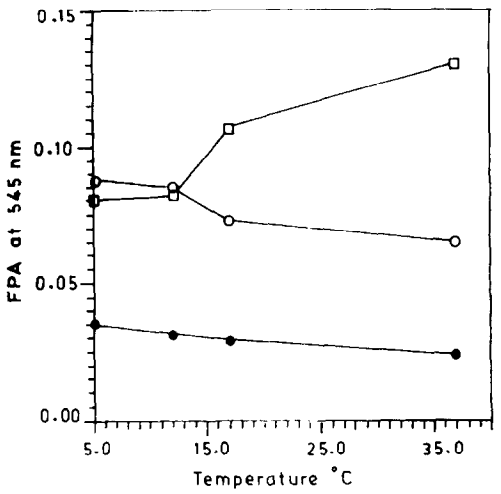

Fig. 4. Changes in fluorescence polarization anisotropy (measured at 545 nm) of TbGTP as a function of temperature under different conditions: (i) alone (–○–○–), (ii) in presence of RNAP (–●–●–) and (iii) in presence of RNAP and pDR720 (–□–□–). Concentrations of TbGTP, RNAP and plasmid were the same as in Fig. 3.

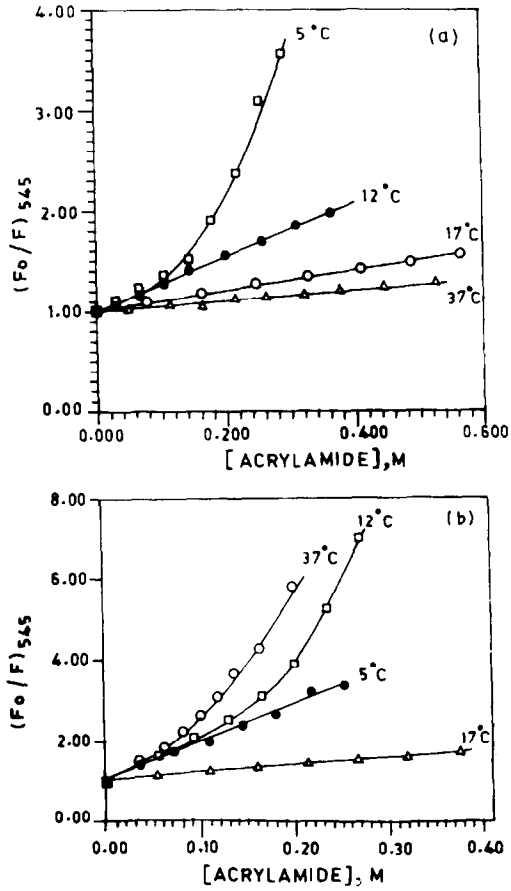

Fig. 5. Stern–Volmer plots for quenching of TbGTP ([Tb(III)] = 77 μM and [GTP] = 12 μM) under conditions (stated below) at different temperatures specified in figures: (a) in the presence of 10 units of RNAP and (b) in the presence of 10 units of RNAP plus 12 nM of pDR720.

that of TbGTP–RNAP. Under the present experimental conditions (legend for Fig. 3), it is unlikely that Tb(III) will dissociate from the complex. We have kept excess RNAP (250 μM in terms of amino acid residues) to avoid formation of any free TbGTP in the system. In absence of any other nucleotide, RNAP is stalled as RP_o at 37°C.

FPA ($\langle r \rangle$) values of TbGTP bound to RNAP both in the absence and presence of template were measured at specified temperatures. In general FPA decreases with temperature due to enhanced Brownian motion at higher temperatures. This is valid for TbGTP alone and TbGTP–RNAP complex as shown in Fig. 4. However, a marked deviation is noticed for TbGTP in its ternary complex with RNAP and promoter DNA. This could be ascribed to a specific feature of the active site of the enzyme, when it is bound to the promoter. It is interesting to note that the deviation starts from 14°C corresponding to formation of RP_{i2}.

Acrylamide quenches the fluorescence of TbGTP complex (figure not shown). This helped us to monitor the extent of quenching of the TbGTP complex when bound to the enzyme, both in the absence and

Table 2
Acrylamide quenching of the TbGTP complex under various conditions in buffer T [a]

Temperature (°C)	TbGTP in presence of RNAP, K_{SV} (M^{-1})	TbGTP in presence of RNAP and *trp* promoter, K_{SV} (M^{-1})
5	3.5	9.5
12	2.8	12.4
17	1.1	1.7
37	0.8	14.7

[a] Acrylamide could quench the free TbGTP complex with a quenching constant of 8.85 M^{-1}, at 26°C.

presence of promoter, under the same experimental conditions where FPA values were estimated. Resulting Stern–Volmer plots are shown in Fig. 5a and 5b. In presence of RNAP alone, there is a decrease in the quenching constant value with temperature. It is typical for static quenching [31]. On the other hand, curves obtained in presence of RNAP and promoter do not follow any general trend, rather the curves have characteristic features. Relevant results are summarized in Table 2. These results also support the earlier proposition that the four complexes possess different active site conformations.

4. Discussion

We have employed a simple fluorescence assay to demonstrate and probe the different steps during initiation of transcription in terms of the conformation of RNAP. Earlier studies proposed a multistep process for transcription initiation by RNAP and defined the open complex (RP_o) in terms of melting of DNA helix around transcription initiation point. Additional footprinting studies have further characterized different intermediate complexes in terms of protection pattern [8–12]. Though subunits of RNAP play cardinal roles along with promoter in formation of these intermediates, yet there are only few studies addressing the conformation of RNAP in these intermediates [15,16,20,21].

First part of our results shows that during $RP_c \rightarrow RP_o$, there are two intermediates with difference in the 3° (or 4°) structures of the protein component in polymerase–promoter complex. The present methodology is not sufficient to detect any change in the 2° structure of RNAP, though the possibility of such change appears remote. We could also demonstrate that there is a conformational transition in the protein due to formation of RP_o from the intermediate RP_{i2}. In the second part, the results of active site probes by the TbGTP complex further suggested that these conformational transitions involve the active site. In these experiments we have compared the fluorescence signal from DNA-bound enzyme with that from the free enzyme to eliminate the error arising from the signal due to any inactive enzyme that is unable to bind the promoter.

4.1. Changes in tertiary / quarternary structure of RNAP during transcription initiation from the trp promoter

Acrylamide, a neutral quenching probe, quenches the tryptophan fluorescence in a purely random and kinetic fashion. Thus, any change in the tertiary/quarternary structure (for a multisubunit protein) leading to an alteration in the accessibilities of these fluorophores could be detected by this sensitive technique. Knowledge of two parameters are necessary for this purpose: quenching constant, K_{SV} (under steady-state conditions) and fraction of accessible tryptophan residues, f_e.

As a control experiment, quenching of fluorescence for free RNAP was also carried out at the same temperatures at which we examined the complexes. Variation of quenching pattern with temperature for free RNAP favors dynamic quenching. There is also no significant change in percentage accessibility of tryptophan residues at four temperatures (Fig. 2). In contrast, we noticed (i) variation of profiles in Stern–Volmer plots for the RNAP–promoter complexes with temperature (figure not shown) and (ii) more remarkably, a significant variation of percentage accessibility of tryptophan residues at four different temperatures (Fig. 2). In order to further emphasize the contrast between the conformation of free and promoter-bound RNAP, ratio of K_{SV}(bound) and K_{SV}(free) is shown in last column of Table 1.

At 5 and 12°C, the nature of the Stern–Volmer plots for the RNAP–promoter complexes is comparable with those for free RNAP. Decrease in accessibility of tryptophan residues from RP_c to RP_{i1} is also not pronounced. This means that gross conformational features of RP_c and RP_{i1} are not radically different. On the other hand, there is a significant decrease in the accessibility of tryptophan residues upon formation of RP_{i2} from RP_{i1}. It implies the onset of conformational changes in promoter-bound holoenzyme during $RP_{i1} \rightarrow RP_{i2}$. The most remarkable conformational feature is exhibited by RP_{i2}. From the drastic change of promoter protection pattern in footprinting studies and non-linear van't Hoff plots for promoter–polymerase interaction [4,9,13], it was proposed that a conformational change in RNAP ensues the nucleation step. Our studies also indicate that in RP_{i2}, conformation of the enzyme has changed

to an appreciable extent leading to static quenching and least accessibility of the tryptophan residues. Probably a hydrophobic pocket is formed in this complex through which acrylamide could penetrate easily. As a result, there is an upward curvature of the Stern–Volmer plot (figure not shown). Formation of hydrophobic pocket in RP_i was also proposed for the transcription initiation from the tetR promoter [9]. There is an increase in accessibility accompanying formation of RP_o, because the tryptophans residues are again aligned on the surface and the distribution is such that most of them (73% of total 14 residues) are equally accessible to acrylamide resulting in a linear Stern–Volmer plot. Neutron scattering studies suggested that the holoenzyme in open complex is a triangular-shaped elongated molecule [16]. This feature leads to a homogeneous population of tryptophan residues on the surface. The holoenzyme has 14 tryptophan residues; ten residues are on β and β' subunits, they also contain the active site of the enzyme [34]. Therefore, it may be proposed that the observed decrease in accessibility of tryptophan residues originates from the conformational changes involving these subunits consequent to their interactions with DNA. Major conformational changes possibly occur in β and β' subunits during the nucleation step, so that the active site is properly formed prior to the open complex formation and start of transcription.

In order to correlate these data with conformational changes in active site structures during transcription initiation, we employed fluorescence labelled substrate TbGTP as a probe in the next part of the study.

4.2. Changes in the active site conformation of RNAP during the process

An earlier study [24] showed that the probe could selectively bind to the substrate binding site (i site) of the enzyme which is situated in the β subunit and about 30 Å away from the rifampicin binding site. Stability of TbGTP complex ($K_d = 0.2$ μM) is greater than that of TbGTP–RNAP ($K_d = 4$ μM) complex. Therefore it is very unlikely that free Tb(III) or the TbGTP complex will be present in the system under our experimental conditions. Changes in FPA and accessibility of the probe to the solvent are good reporters of the events occurring at the active centre during the initiation process. Acrylamide has been used as a quencher to measure the accessibility of TbGTP to the solvent because it quenches fluorescence of TbGTP complex ($K_{SV} = 8.85$ M^{-1} at 26°C) and it is a non-invasive substance for RNAP conformation or activity within the range of concentrations employed here.

The anisotropy value for TbGTP in presence of enzyme alone is relatively low and decreases with temperature. It originates from the rotational freedom of TbGTP within the protein matrix. This implies that the phosphate groups of the probe do not interact directly with the amino acid residues; rather, they remain away from the active site. The proposition is also consistent with earlier observations [35,36]. FPA values for RP_c and RP_{i1} are comparable with the value for the complex with free protein. It means that the gross conformation of the active site has not undergone any drastic change from that in the free protein. On the other hand, FPA increases significantly in the steps $RP_{i1} \rightarrow RP_{i2}$ and $RP_{i2} \rightarrow RP_o$, exhibiting the highest value at RP_o (Fig. 4). It implies that through the intermediate stages, RNAP in an open complex has attained a conformation where substrate is in a most rigid environment. An earlier NMR study showed that in the presence of DNA, the phosphate groups of the initiating nucleotide moves closer (about 3 Å) to the metal ion at the active centre [35]. Probably this conformational change in active site during formation of RP_o is needed for proper geometric orientation of the initiating nucleotide with the incoming 3′-OH group of following substrate at i + 1 site as well as with the template.

TbGTP in free protein exhibits static quenching, because K_{SV} decreases with temperature. A relatively high concentration of acrylamide was needed to achieve the quenching at all temperatures, both in presence and absence of the promoter. From the genetic studies [37], it has been postulated that the active centre lies within a well-defined cleft at the interface of all the subunits and it is relatively sequestered from the solvent. Our data also suggest that the TbGTP binding site is within a hydrophobic pocket. It is also relatively less accessible to acrylamide due to presence of the promoter.

The presence of promoter DNA leads to an increase in the K_{SV} value of the TbGTP complex in

all intermediate complexes. It indicates that the promoter induces a gradual 'opening up' of the hydrophobic pocket towards the solvent. Each intermediate has a characteristic Stern–Volmer profile as well as the dynamic and static quenching values corresponding to distinguishable conformational states of RNAP. Accessibility of TbGTP increases gradually upto the formation of RP_{i1} with significant static quenching. It reduces at 17°C upon the formation of RP_{i2} and becomes most accessible in RP_o. This signifies two distinct classes of conformations in RNAP. The binding cleft opens up to some extent without gross change in protein structure until RP_{i1} is formed. A significant subunit rearrangement may occur in the following steps, $RP_{i1} \rightarrow RP_{i2}$ and $RP_{i2} \rightarrow RP_o$. RP_{i2} is a potential intermediate in this reshuffling process. The active center in RP_{i2} transiently goes into the protein matrix and as a result it becomes less accessible to acrylamide. This proposal agrees with the recent finding of major change in cross-linking pattern (from $\beta\sigma$ and $\beta\beta'$ to only $\beta\beta'$) during formation of RP_i [21]. The maximum accessibility of the active site in RP_o indicates an optimized conformation for facile entry of substrates and also for the accommodation of 9 base long nascent RNA chain [38]. During the whole process, nucleic acid framework along with the enzyme plays an important role [39]. Thus major conformational changes in RNAP occur in the active site during the process of transcription initiation.

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