Enhancement of transcriptional activity of T7 RNA polymerase by guanidine hydrochloride

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Received 31 March 1998

Abstract T7 RNA polymerase shows an increase in processive transcription in the presence of low concentrations of guanidine hydrochloride (GdnCl) upto 60 mM, which is not observed when the enzyme is treated with urea. Higher concentrations of the denaturant lead to a progressive loss in the processive transcriptional activity of the enzyme. We have attempted to explain the above phenomenon in terms of the structural change in the enzyme. Fluorescence and CD studies suggest that the tertiary structure of the native enzyme undergoes an alteration upon addition of low concentration of guanidine hydrochloride. This is also indicated from the decreased susceptibility of the enzyme to limited proteolysis by trypsin.

Key words: T7 RNA polymerase; Guanidine hydrochloride; Processive transcription; Abortive transcription

1. Introduction

Bacteriophage T7 RNA polymerase (T7 RNAP) is a single polypeptide chain of 883 amino acids (molecular weight 99 kDa), having stringent specificity for its cognate promoter [1–3]. One approach to study the structure and function of a protein and its folding pathways is to examine the effect of denaturants on it [4]. So far, there have been no studies on the effect of denaturants like urea or guanidine hydrochloride (GdnCl) on the structure and activity of T7 RNAP. In the present study, we have examined the effects of the denaturants, guanidine hydrochloride and urea, upon the transcriptional activity of T7 RNAP. The significant observation from these studies is that low concentration ($\sim$50 mM) of guanidine hydrochloride enhanced the transcriptional activity of T7 RNAP as measured from the production of full length transcripts. On the other hand, the enzyme activity in terms of full length transcripts was absent at about 300 mM guanidine hydrochloride. This is not observed when the enzyme is treated with urea, where the denaturation profile follows the normal two-state model observed with the majority of functional proteins [5,6]. Simultaneously, we have probed the structural perturbation in the enzyme under the influence of guanidine hydrochloride by means of fluorescence and CD spectroscopy with a view to correlate the above observation with the structure of the enzyme. The structure is also probed from a comparison of the kinetic profile for the limited proteolysis of the enzyme alone and in presence of 50 mM guanidine hydrochloride. Results from these studies indicate an alteration in the tertiary structure of the protein as the cause leading to the aforementioned enhanced activity of the enzyme. Plausible interpretation of the above phenomenon in terms of guanidine hydrochloride induced transition from abortive cycling to processive transcription is discussed.

2. Materials and methods

Guanidine hydrochloride, urea, acrylamide, magnesium chloride, Tris, ethylenediaminetetraacetic acid (EDTA), glycerol, dithiothreitol (DTT), trypsin, trypsin inhibitor and spermidine were from Sigma Chemical Co. (St. Louis, MI, USA); nucleoside triphosphates (ATP, GTP, CTP and UTP) of high purity were from Pharmacia Fine Chemicals, Sweden. Media for bacterial growth were from Difco Laboratories, USA. [α-^{32}P]-UTP was from BRIT, India. Rest of the chemicals used were of analytical grade. T7 RNAP was isolated from the overproducing *Escherichia coli* strain BL21, harboring the plasmid *pAR1219* that has the polymerase gene cloned under *lac* UV5 promoter [7]. The strain was kindly provided by Prof. F.W. Studier, Brookhaven National Laboratories, New York, USA. The enzyme was isolated according to the method described by Grodberg and Dunn [8] and purified by a modified protocol developed in our laboratory. Purity of the enzyme was confirmed from the presence of a single band on SDS-PAGE and concomitant enzyme activity. All experiments were done in buffer prepared with double distilled deionized water.

2.1. Activity assays

T7 RNAP was assayed by in vitro transcription in a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM DTT, 10 mM $MgCl_2$ and 4 mM spermidine. The transcription mixture contained Φ10 promoter-containing plasmid DNA (linear or circular) at a concentration of 50 μg/ml and 1 mM of each NTP along with 1 μCi of α-[^{32}P]-UTP (specific activity 3 Ci/μM). The enzyme (1.0 μM) was incubated with different concentrations of GdnCl or urea for 30 min at 37°C (or for 1.5 h at 20°C) and then added to the transcription mixture to a final concentration of 0.5 μM. After incubation of the mixture for 10 min at 37°C, the reaction was stopped by freezing to −20°C and the assay was carried out according to standard method by filter binding assay [9]. Percentage incorporation was calculated from the ratio of the incorporated count and total count, which gives a measure of the full length transcripts formed. Short length abortive transcripts formed during transcription were analyzed by autoradiography as follows. After transcription, the reaction was terminated by adding 90% formamide-containing loading dye, boiled for 5 min and then the mixture was loaded to a 25% polyacrylamide/7 M urea gel. It was electrophoresed and autoradiographed according to standard methods.

2.2. Spectroscopic studies

A fixed concentration (0.5 μM) of the enzyme was incubated separately with different concentrations of GdnCl for 1.5 h at 20°C to examine the effect of GdnCl treatment on the intrinsic fluorescence of T7 RNAP. Then the fluorescence emission spectra of the enzyme excited at 295 nm (or 278 nm) were recorded in a Hitachi F4010 spectrofluorometer, at excitation and emission band passes of 5 nm and 10 nm, respectively. Contribution from the TDMK buffer (50 mM Tris-HCl, pH 8.0, containing 50 mM KCl, 10 mM $MgCl_2$ and 1.24 mM DTT) and GdnCl was subtracted whenever required.

CD spectral change of the protein upon GdnCl treatment was fol-

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PII S0014-5793(98)00458-X

lowed in far (200 nm to 250 nm) and near (250 nm to 310 nm) UV regions using a Jasco J-720 spectropolarimeter. They were recorded in 1 mm (far UV) and 1 cm (near UV) path length cuvettes, respectively. The recorded spectra are an average of three runs and appropriately subtracted for contribution from the TDMK buffer with or without GdnCl.

2.3. Limited proteolysis

The native enzyme alone and after treatment with 50 mM GdnCl for 1.5 h at 25°C were separately subjected to proteolysis by trypsin (T7 RNAP: trypsin in 640:1 w/w ratio) in 20 mM Tris-HCl buffer containing 1 mM EDTA and 100 mM NaCl at 30°C. At the indicated time intervals, aliquots were removed and the reaction stopped by adding excess bovine pancreatic trypsin inhibitor [10]. These were then subjected to electrophoresis in denaturing 7.5% polyacrylamide gels. The gel was stained by coomassie blue.

3. Results

3.1. Effect of GdnCl on the transcriptional activity of T7 RNAP

Full length transcriptional activity of the enzyme as a function of the input concentrations of GdnCl or urea is shown in Fig. 1. The activity of the untreated enzyme was taken as 100% and activities of the treated enzyme were calculated relative to that of the native enzyme as follows: (activity of the treated enzyme/activity of the native enzyme)×100. Low concentrations of GdnCl (less than 60 mM) induce an increase in the activity of T7 RNAP relative to that of the untreated enzyme, with maximum enhancement occurring around 50 mM GdnCl. Thereafter, the activity fell rapidly with increasing concentrations of GdnCl and it was completely abolished at around 300 mM GdnCl. The increase is a novel feature of our observation. The above nature of the denaturation profile is absent when urea is used as the denaturant. In this case, the loss of activity follows the normal two-state (N⇔D) process observed when most of the proteins are treated with denaturants [5,11].

Since T7 RNAP is well known to produce short length

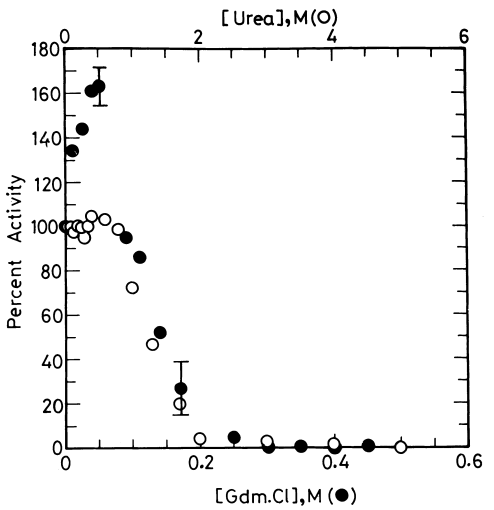

Fig. 1. Percentage activity of T7 RNA polymerase (0.5 μM) in terms of full length transcription at 37°C, as a function of the input concentrations of denaturants, guanidine hydrochloride (●) and urea (○), respectively. The error bars are shown for two representative sets. The experiments were done in transcription buffer, 20 mM Tris-HCl, containing 1 mM DTT, 10 mM $MgCl_2$ and 4 mM spermidine, pH 8.0, with four different batches of the enzyme. Percentage activity has been calculated by the method described in the text.

Fig. 2. Autoradiogram of abortive transcription products formed by T7 RNAP under different conditions, resolved in 25% polyacrylamide/7 M urea gel: Lane 1, native T7 RNAP; lane 2, T7 RNAP treated with 50 mM GdnCl; lane 3, T7 RNAP treated with 500 mM GdnCl. The uppermost band in lanes 1 and 2 represents the full length transcripts which remain in the groove in 25% polyacrylamide gel.

abortive transcripts which are not detected in the filter binding assay mentioned above, the transcription products were analyzed by polyacrylamide gel electrophoresis to check for the small length RNAs. Fig. 2 shows the autoradiogram of polyacrylamide gels to resolve such transcripts. The uppermost band represents the full length transcripts present in the groove, since they are unable to enter the 25% gel. The absence of such a band in case of treatment with 500 mM GdnCl indicates that processive transcription by T7 RNAP does not occur under that condition. On the other hand, the lower bands indicate that abortive transcription occurs even upon treatment of the enzyme with 500 mM GdnCl. The relative intensity of the bands due to short length RNA transcripts apparently decreases in the presence of 50 mM GdnCl as compared to the native protein, giving way to an increase in the full length products. Such change in the pattern of the catalytic function of the enzyme in the presence of low concentration of GdnCl would originate from the denaturant induced alteration in the structure of the enzyme. Therefore, the structure of the enzyme was probed by fluorescence and CD spectroscopic methods.

3.2. Effect of GdnCl on the structure of T7 RNAP

Fig. 3a and b show the representative fluorescence emission spectra of the native enzyme and enzyme treated with different concentrations of GdnCl, upon excitation at 295 nm and 278 nm, respectively. The increase in the emission intensity at 340 nm upon treatment with 50 mM GdnCl is a distinctive feature. This increase is observed at both excitation wavelengths, 278 nm and 295 nm, thereby confirming it. Subsequent decrease in the emission intensity at higher concentrations of GdnCl and the accompanying red shift of the peak in presence of 3 M GdnCl is consistent with the general trend reported for

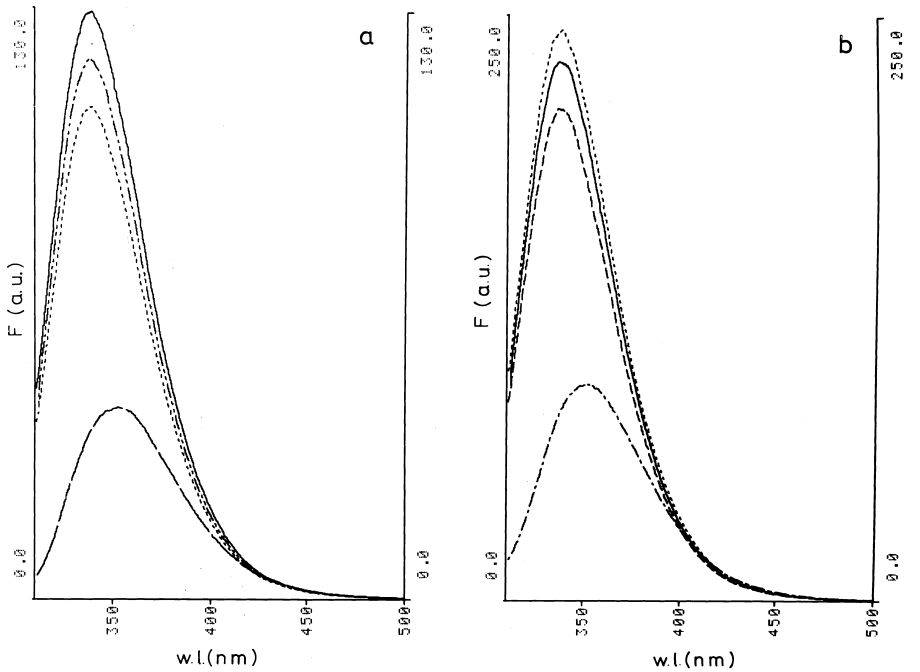

Fig. 3. Representative fluorescence emission spectra at 25°C, of T7 RNA polymerase (0.5 μM) in TDMK buffer, pH 8.0 upon treatment with 50, 0, 500 mM and 3.0 M guanidine hydrochloride, respectively, in decreasing order of emission intensities. a: Excitation wavelength = 295 nm, b: excitation wavelength = 278 nm.

other proteins [12]. The increase in fluorescence at 50 mM GdnCl originates from the change in tertiary structure of the enzyme resulting in the altered electronic environment of the fluorophore.

CD spectra of native and GdnCl treated T7 RNAP in the far and near UV regions are shown in Fig. 4. The far UV CD spectrum of native T7 RNAP showed predominantly alpha helical characteristics with two negative bands at 209 nm and 222 nm. The calculated helical content of the protein from the observed values of molar ellipticities is consistent with reported values for the native protein [13]. When the protein was treated with 50 mM GdnCl, the negative ellipticities at 209 nm and 222 nm did not decrease. Instead, there is a marginal increase in the above ellipticity values. On the other hand, addition of 3 M GdnCl leads to a total loss in the helical content as expected. Thus the backbone or secondary structure of the enzyme is apparently stabilized on treatment with low concentration of GdnCl. The CD spectrum of the native T7 RNAP in the near UV region has a positive band around 290 nm. It is contributed by the side chains of the large number of aromatic amino acids present in the enzyme. On treating with 50 mM GdnCl the amplitude of the positive band in the CD spectrum of the enzyme increases. There is also a concomitant change in the fine structure profile of the CD spectrum in comparison to that of the native protein. Change in the near UV CD spectrum originates from the

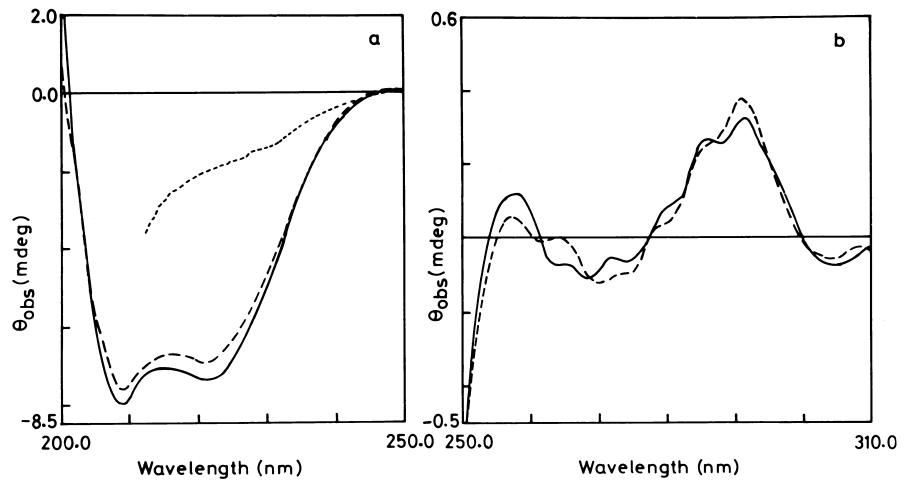

Fig. 4. a: Far UV (200–250 nm) CD spectra of 0.5 μM T7 RNA polymerase alone (dashed line with long dashes) and upon treatment with 50 mM (line) and 3.0 M (dashed line with short dashes) GdnCl, respectively. Experiments were done in TDMK buffer, pH 8.0, at 25°C. b: Near UV (250–310 nm) CD spectra of 1.0 μM T7 RNA polymerase alone (line) and upon treatment with 50 mM GdnCl (dashed line). Experiments were done in TDMK buffer, pH 8.0, at 25°C.

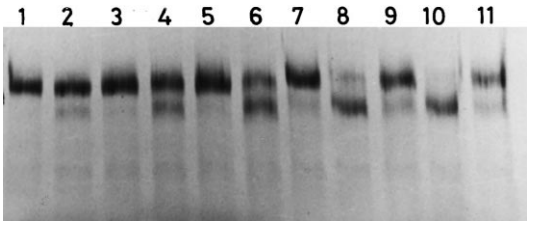

Fig. 5. SDS-PAGE analysis of the products formed on limited trypsinization of free T7 RNAP (1.0 μM) and T7 RNAP (1.0 μM) treated with 50 mM GdnCl. Lanes 1, 2, 4, 6, 8 and 10 are the products formed after 0, 2.5, 5.0, 10.0, 20.0 and 30.0 min of trypsin digestion of free T7 RNAP. Lanes 3, 5, 7, 9 and 11 show the products formed after 2.5, 5.0, 10.0, 20.0 and 30.0 min of trypsin digestion of T7 RNAP pre-treated with 50 mM GdnCl. Conditions of proteolysis are given in the text.

alteration of tertiary structure in the enzyme [14]. Having detected a change in the structure of the protein upon treatment with 50 mM GdnCl, we attempted to provide further evidence for such change by biochemical method of limited proteolysis [10,15].

The region between amino acid residues 170–180 in T7 RNAP is susceptible to trypsin digestion. It leads to the formation of 80 kDa and 20 kDa peptide fragments [10]. Fig. 5 shows the coomassie stained polyacrylamide gel indicating the difference in susceptibility of T7 RNAP to trypsin digestion when treated with 50 mM GdnCl. The figure shows that the native enzyme is cleaved fully to the 80 kDa fragment in 30 min. In contrast, GdnCl treated enzyme just starts to get cleaved at this time point. It is, therefore, evident that the conformational change induced by 50 mM GdnCl in T7 RNAP reduces its susceptibility to proteolysis as reflected from the longer time required for the cleavage by trypsin.

4. Discussion

The effect of GdnCl treatment on the structure and function of T7 RNAP has been probed in these studies. The transcriptional activity enhanced on treatment with low concentrations of GdnCl (less than 60 mM), whereas there was lesser formation of abortive transcripts. It is a novel feature hitherto unreported for any other enzyme. This could be ascribed to a change in the tertiary structure of the protein. The increase in the fluorescence quantum yield and alteration of the near UV CD spectrum of the enzyme in the presence of 50 mM GdnCl support the proposition for a change in tertiary structure. Alteration in the tertiary structure is also indicated from the reduced sensitivity of the enzyme to limited proteolysis by trypsin in the presence of 50 mM GdnCl. The above observation could not be ascribed to a salt effect because high concentration of sodium chloride (100 mM) does not exert any influence upon the activity of the enzyme, nor does it alter the spectroscopic properties of the enzyme in a similar fashion.

A plausible explanation in terms of the transcriptional activity of the enzyme could be forwarded as follows. Low concentration of GdnCl induces a conformational change in T7 RNAP such that the transition from abortive cycling to processive transcription is favored. Earlier reports have shown that the thumb subdomain of T7 RNAP (helix O, residues 370–405, [16]) can bend about residue 388 to wrap around the DNA template and stabilize the ternary complex, T7 RNAP-DNA-RNA. This leads to a transition from abortive cycling to processive transcription [17,18]. Such a bending might be facilitated upon treatment with low concentration (50 mM) of GdnCl, thereby increasing the extent of processive transcription with concomitant decrease in abortive transcription. It is also indicated from the conformational changes of the enzyme detected spectroscopically and biochemically in the present study.

This unique observation may have an important practical application. Since T7 RNAP is widely used for in vitro transcription studies and protein overexpression systems, addition of a low concentration of GdnCl will enhance its activity and help in greater production of the proteins.

Acknowledgements: We thank Prof. S.K. Ghosh of Crystallography and Molecular Biology Division of our institute for making the Jasco J-720 spectropolarimeter available.

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