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Structural studies of arginine induced enhancement in the activity of T7 RNA polymerase

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ABSTRACT

Addition of arginine enhances the activity of the enzyme T7 RNA polymerase. Different methods have been employed to understand the enhancement in the light of arginine induced alteration of the tertiary structure. The increase in activity of the enzyme reaches a maximum value around a concentration of 125 mM arginine. Fluorescence, circular dichroism and dynamic light scattering studies indicate an alteration in the tertiary structure of the enzyme. Enthalpy change as a function of input concentration of arginine to a fixed concentration of the enzyme (5 μM) shows a dip at 100 mM concentration of arginine. Differential scanning calorimetric studies of the denaturation of the enzyme in absence and presence of arginine indicates arginine induced destabilization of the C-terminal domain of the enzyme. Structural alterations induced by arginine have been compared with those induced by the denaturant guanidine hydrochloride.

1. Introduction

Bacteriophage T7 RNA polymerase (T7 RNAP), a single polypeptide chain of 883 amino acid residues (molecular weight 99 kD), has a stringent specificity for its cognate promoter, and can carry out promoter-specific initiation, elongation and termination of transcription without the help of any accessory factor *in vitro*. The transcriptional mechanism of T7 RNAP is apparently similar to that of multisubunit RNA polymerases (RNAP) such as *Escherichia coli* RNAP. The relative simplicity of single subunit phage T7 RNAP as compared to its bacterial and eukaryotic counterparts makes this enzyme a good model for studies on RNA polymerase structure and function, especially to understand the basis of polymerase–promoter interaction [1–3] and the mechanism of transcription [4–7].

In the present report we have probed the structural perturbation of T7 RNA polymerase by arginine, an amino acid known for its ability to suppress aggregation of certain proteins [8] and to enhance the chaperone like activity of α-crystallin [9]. Our present report consists of three aspects. First, we have demonstrated the enhancement of transcriptional activity of the enzyme upon addition of the amino acid. Secondly, fluorescence, circular dichroism and dynamic light scattering studies have been carried out to study the nature of the alteration of structure and hydrodynamic diameter of the enzyme as a sequel to the addition of the amino acid. We have also characterized the structural alteration in terms of the associated thermodynamics of the process. Finally, the results have been compared with structural perturbations induced by low concentration of guanidine hydrochloride. The studies have helped to know the nature of arginine induced structural perturbation of the enzyme and the role played by the amino acid arginine. The observation reported here expands the application potential of arginine for biotechnology [10]. This is also the first report showing arginine induced increase in activity of any enzyme.

2. Materials and methods

2.1. Chemicals

Guanidine hydrochloride, L-arginine, magnesium chloride, tris, EDTA, HEPES, glycerol, IPTG and dithiothreitol were from Sigma Chemical Company (USA). Media for bacterial growth were purchased from HiMedia (India).

2.2. Isolation and purification of T7 RNA polymerase

T7 RNAP was isolated according to the method described by Grodberg and Dunn [11], and purified by a method developed in

Abbreviations: T7 RNAP, T7 RNA polymerase; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; EDTA, ethelenediaminetetraacetic acid; ATP, adenosine triphosphate; CTP, cytosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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our laboratory [12]. Prior to experiments, the T7 RNAP was extensively dialyzed against the appropriate buffer. The purity of the enzyme preparation was checked by SDS–PAGE. The concentration of T7 RNAP was determined from its absorbance at 280 nm using the molar extinction coefficient value of $\varepsilon = 1.4 \times 10^5\ M^{-1}\ cm^{-1}$[13].

2.3. Activity assays

The activity of T7 RNAP was checked by *in vitro* transcription in transcription buffer containing 20 mM Tris–HCl (pH 8.0), 1 mM DTT, 10 mM $MgCl_2$ and 4 mM spermidine. The transcription mixture consisted of 10 promoter containing plasmid DNA (either circular or linearized by restriction enzyme digestion) at a concentration of 50 μg/ml, 1 mM each of UTP, GTP, CTP and ATP along with 0.25 μCi of -[^{32}P]-UTP (specific activity 3000 Ci/mM). 1 micro molar enzyme was incubated with different concentrations of arginine for 1.5 h at 20 °C (or for 30 min at 37 °C) and then added to the transcription mixture to a final concentration of 0.5 μM. Transcription was allowed to occur for 10 min at 37 °C. The reaction was then stopped by freezing the mixtures to −20 °C and a standard filter binding assay was carried out [5,14]. The transcription mixtures were thawed on ice and spotted onto two glass fiber (GF/C) filters each, and allowed to dry. One of the filters was washed with 5% TCA solution, followed by washing both filters with 70% ethanol. They were dried, dipped in the scintillation fluid, Cocktail 'O', and the radioactive counts for each were counted using a LKB 1211 Rack Beta liquid scintillation counter. TCA unwashed filters gave the total applied radioactivity, while the washed filters gave the amount of radiolabel incorporated into the transcribed RNA, which was retained on the filter as acid precipitable material. Percentage incorporation was calculated from the ratio of the incorporated count and total count, which gave a measure of the full length transcripts formed. The activity of the untreated enzyme was taken as 100% and the percent activities of the treated enzyme were calculated relative to that of the native enzyme.

% activity of the treated enzyme = (activity of the treated enzyme/activity of the untreated enzyme) × 100.

2.4. Steady-state fluorescence spectroscopy

A fixed concentration (0.5 μM) of the enzyme in TDMK buffer (50 mM Tris–HCl (pH 8.0) containing 50 mM KCl, 10 mM $MgCl_2$, 1.24 mM DTT) was incubated separately with different concentrations of arginine for 1.5 h at 20 °C to examine the effect of such treatment on the intrinsic fluorescence of the enzyme. The emission spectra of tryptophan residues of the enzyme, selectively excited at 295 nm (or all aromatic residues excited at 278 nm), were then recorded in a Hitachi F4010 spectrofluorimeter, with excitation and emission band passes of 5 and 10 nm, respectively. Corrections due to inner filter effect were not required as the absorbance of the samples in TDMK buffer did not exceed 0.05. The emission intensity at 340 nm was plotted as a function of arginine concentrations.

2.5. Isothermal titration calorimetry (ITC)

Interaction of T7 RNAP with arginine/guanidine hydrochloride was studied using MicroCal iTC200 (MicroCal, USA). ITC experiments were carried out in 20 mM HEPES buffer (pH 8), containing 100 mM NaCl, 5% glycerol, 1 mM EDTA, 0.5 mM β-mercaptoethanol and 10 mM $MgCl_2$. Five micro molar of the protein solution, in the same buffer was taken in the cell. 1.5 M solution of arginine/guanidine hydrochloride in the same buffer was added from the syringe. The heat of dilution of the ligand was measured by a control experiment where only arginine/guanidine hydrochloride was injected into the buffer. The heat of dilution was subtracted form the data obtained after titration of the ligand with the protein. The data was analyzed using the inbuilt MicroCal Origin software of the calorimeter. Results from the above experiments show that the enthalpy change for arginine is maximum in the concentration range of 100–150 mM. We have therefore used 100 mM arginine as a representative concentration in the subsequent experiments.

2.6. Dynamic light scattering

Dynamic light scattering studies were carried out using Zetasizer NanoS particle analyzer from Malvern Instruments Ltd., UK. The Zetasizer measures the fluctuation in scattering intensity and determines the diffusion coefficient, *Q*, of the sample by means of its inbuilt auto-correlator. Size of the particles is calculated using Stokes–Einstein equation:

$$D_H = \frac{kT}{f} = \frac{kT}{3\pi\eta Q}$$

where, D_H is the hydrodynamic diameter, k is the Boltzmann constant, f is the particle frictional coefficient, η is solvent viscosity, T is the absolute temperature and Q is the diffusion coefficient, as calculated by the inbuilt auto-correlator. All sample solutions were filtered through a 0.22 μm filter (Millipore, USA).

The scattering intensity of T7 RNAP alone and in presence of L-arginine/guanidine hydrochloride were monitored in 20 mM HEPES, pH 8.0 at 20 °C. Ten measurements of 12 runs each were averaged by its inbuilt software (Dispersion Technology Software Version 4.10) to give a statistical value of the diameter size (nm) of the particles present in the solution.

2.7. Circular dichroism spectroscopy

Conformational change of T7 RNAP upon interaction with arginine was also probed by CD spectroscopy using a Jasco J720 spectropolarimeter. Far UV CD spectra (250–202 nm) of T7 RNAP incubated with L-arginine could not be measured because of high value of absorbance of L-arginine in this region. Tertiary structural change was probed by recording CD spectra of 4 μM T7 RNAP either free, or after treatment with 100 mM arginine, in the near UV region (310–250 nm). All spectra were recorded in TDMK buffer, pH 8.0, at 25 °C and an average of four scans were reported, smoothened to permissible limits by the inbuilt software of the instrument.

2.8. Differential scanning calorimetry (DSC)

Differential scanning calorimetry experiments to investigate thermal stability of T7 RNA polymerase in presence of arginine/guanidine hydrochloride were performed using a VP-DSC Microcalorimeter, MicroCal, USA. Data were collected at a heating rate of 60 °C/h. The experiments were performed in 20 mM HEPES, pH 8.0 containing 100 mM NaCl, 5% glycerol, 10 mM $MgCl_2$, and 0.5 mM β-mercaptoethanol. Five micro molar of T7 RNA polymerase was incubated with 100 mM arginine/50 mM guanidine hydrochloride for 1.5 h at 20 °C. Then the samples were loaded onto sample cell of the calorimeter. DSC curves were analyzed using the inbuilt MicroCal Origin software, provided with the instrument.

3. Results

The activity profile of T7 RNAP as a function of arginine concentration shows a broad peak around the concentration range of 100–150 mM of arginine with an increase in activity by about 230% in

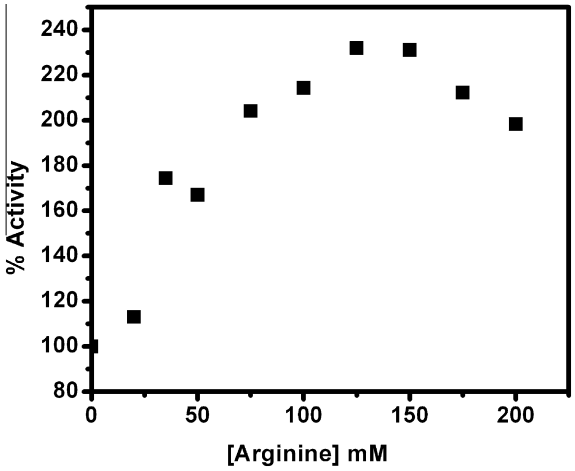

Fig. 1. Activity profile of T7 RNAP as a function of concentration of arginine.

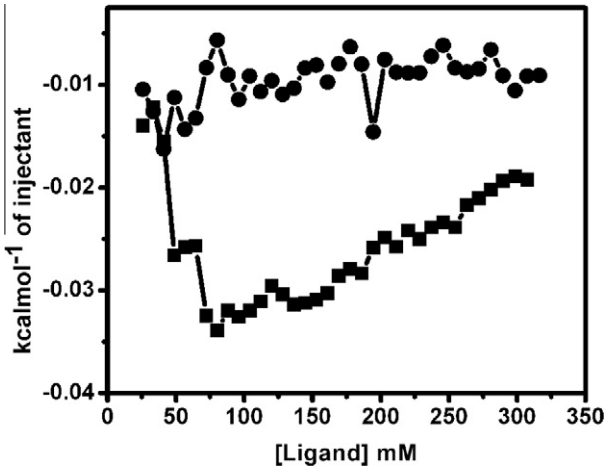

Fig. 3. Isothermal titration calorimetric data for titration of T7 RNAP with (●) guanidine hydrochloride and (■) arginine in 20 mM HEPES, pH 8, 100 mM NaCl, 5% glycerol, 1 mM EDTA, 0.5 mM β-mercaptoethanol and 10 mM $MgCl_2$ at 20 °C. The titration profiles were obtained after subtracting the effect of guanidine hydrochloride/arginine dilution.

presence of 125 mM arginine (Fig. 1). We have previously shown that low concentration of guanidine hydrochloride (50 mM) also substantially increases (by about 160%) the activity of T7 RNAP [15]. Our results suggest that arginine is more efficient than guanidine hydrochloride in increasing the enzymatic activity of T7 RNAP. Enhancement of enzymatic activity in presence of both ligands indicates that the common guanidinium group might facilitate the activity enhancement. Therefore, we have compared the effects of guanidine hydrochloride and arginine upon the structure and conformation of the enzyme.

Fig. 2A shows that in presence of arginine, fluorescence emission intensity of T7 RNAP at 340 nm increases upon excitation at both 278 and 295 nm. The increase in quantum yield of the enzyme indicates a change in the environment of the aromatic amino acids. Similar increase was also reported in case of guanidine hydrochloride [15]. Addition of 100 mM arginine shows the following changes in the near UV CD spectrum of T7 RNAP (Fig. 2B): increase in peak intensity at 292 nm, the broadening of the shoulder present at 282 nm and enhancement of the small peak at 258 nm. Such changes in CD signal originate from alteration in the environment of aromatic amino acids as a result of change in tertiary structure. However, these changes in CD spectrum do not overlap with guanidine hydrochloride induced change [15].

Isothermal titration calorimetry (Fig. 3) shows the dependence of enthalpy change upon ligand concentration. Initial decrease in the enthalpy at lower concentration of arginine suggests predominance of electrostatic interaction between arginine and the negatively charged amino acid residues on the surface of the protein. The decrease is followed by a characteristic trough around the region of 75–150 mM arginine. A similar dip has been reported in case of interaction of n-alkyl sulfates with cytochrome c at pH 2 [16]. Such a dip has been ascribed to the formation of an energetically stabilized partially unfolded intermediate. In contrast, no such dip is observed in case of guanidine hydrochloride. It may be proposed that interaction between T7 RNAP with arginine leads to an energetically stabilized state of the protein at this range of arginine concentrations. The enhancement of enzymatic activity in presence of arginine can be a consequence of the formation of such an energetically favorable intermediate structure of T7 RNAP. At higher concentrations, arginine has a propensity to form molecular clusters and it interacts with proteins using the hydrophobic surfaces [17]. Steady increase of enthalpy at higher concentrations (>150 mM) of arginine, therefore, might originate from a change-

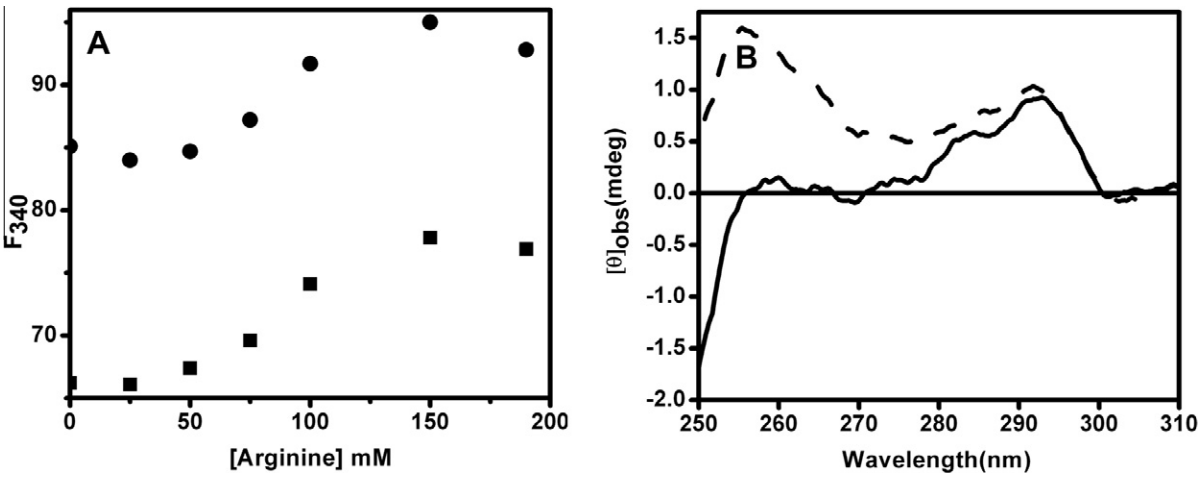

Fig. 2. Arginine induced perturbation of the environment of aromatic amino acids in T7 RNAP. A: Fluorescence emission intensity of 0.5 μM T7 RNAP at 340 nm as a function of arginine concentration. Excitation wavelengths were 295 nm (■) and 278 nm (●) B: Near UV (310–250 nm) CD spectra of native T7 RNAP(4 μM, solid line) and T7 RNAP incubated with 100 mM arginine for 1.5 h (dashed line).

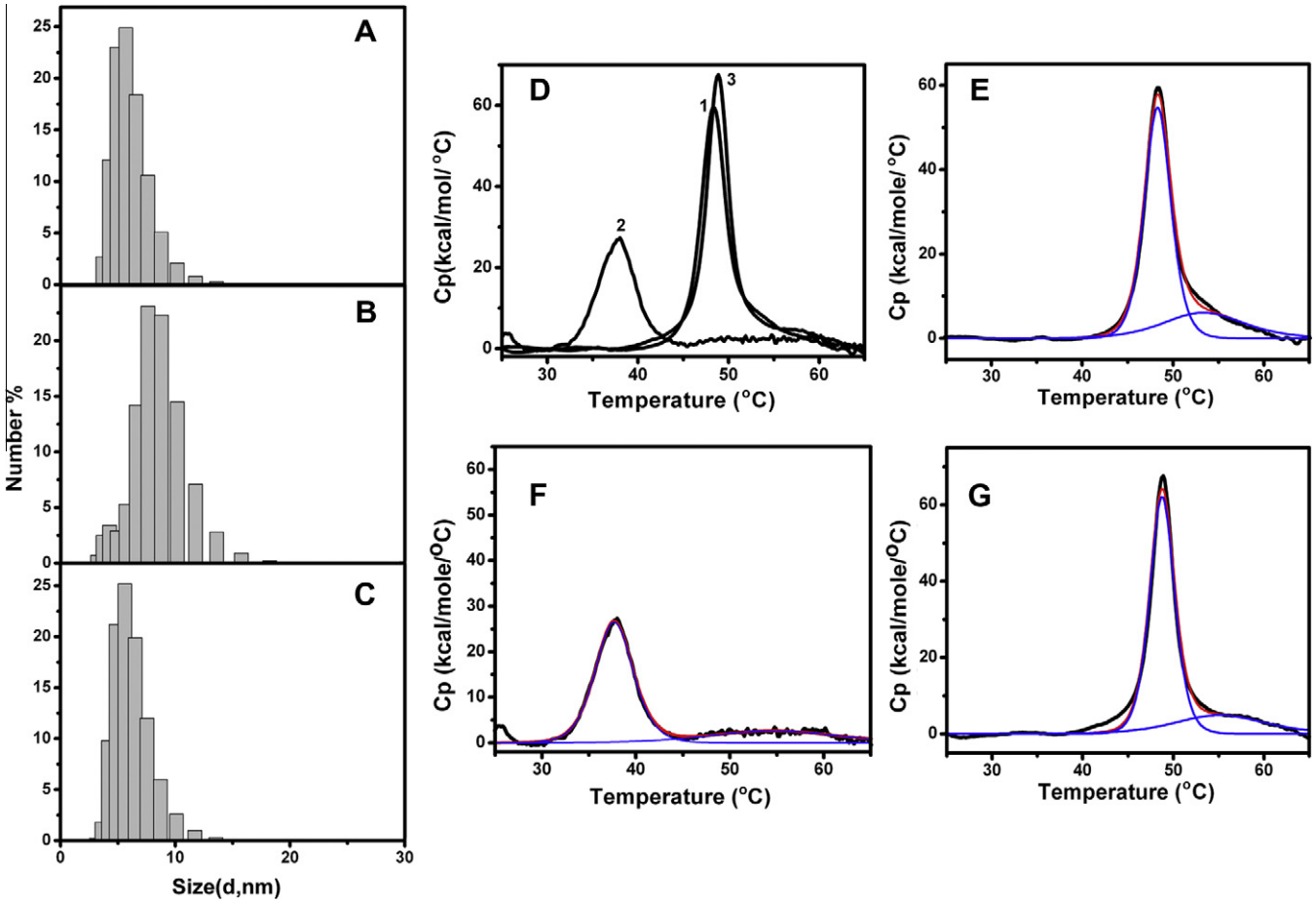

Fig. 4. Effect of arginine/guanidine hydrochloride on the hydrodynamic diameter and domain structure of T7 RNAP. Distribution of the number of particles (in percent) of T7 RNAP as a function of size(diameter in nm)obtained from dynamic light scattering: Native T7 RNAP(5 μM) (A), T7 RNAP incubated with 100 mM arginine for 1.5 h at 20 °C (B), T7 RNAP incubated with 50 mM guanidine hydrochloride (C). Deconvolution analysis of the heat capacity function of T7 RNAP in different conditions, (D) excess heat capacity function of native T7 RNAP (1), T7 RNAP treated with 100 mM arginine (2), T7 RNAP treated with 50 mM guanidine hydrochloride (3). The blue lines show the components obtained after deconvolution of the excess heat capacity function of native enzyme (E), T7 RNAP treated with 100 mM arginine (F), T7 RNAP treated with 50 mM guanidine hydrochloride (G) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

over from hydrophilic nature to hydrophobic nature of the interaction [18]. The difference in the ITC profiles between arginine and guanidine hydrochloride suggests that guanidinium group is not the exclusive factor for the increase in activity.

The hydrodynamic diameter of the protein increases from 5.6 nm for native protein to 8.7 nm for T7 RNAP treated with 100 mM arginine (Fig. 4A and B). In contrast, the hydrodynamic diameter of T7 RNAP remains unaltered in presence of 50 mM guanidine hydrochloride (Fig. 4C). Fig. 4D shows the variation of excess heat capacity of T7 RNAP with temperature in absence and presence of 100 mM arginine/50 mM guanidine hydrochloride. Deconvolution of the heat capacity function of T7 RNAP demonstrates the presence of overlapping two state transitions (Fig. 4E). They originate from the two domains in T7 RNAP, the C-terminal domain and the N-terminal domain. We have assigned the peaks at 48.3 °C (Transition I) and 53.4 °C (Transition II) to C-terminal and N-terminal domains, respectively (Table 1) from a comparison with a previously reported profile of T7 RNAP [19]. We have not considered another related report [20], because it was done in a different buffer and pH. The currently reported melting temperature of 53.4 °C for N-terminal domain differs from the previously reported value of 44 °C [19]. The difference may be attributed to the stabilizing effect of glycerol used in our studies. Glycerol suppresses the flexibility of protein structures and its presence in the experimental buffer might be the key factor in stabilizing the conformationally flexible N-terminal domain of T7 RNAP [21]. Addition of arginine results in a decrease in the melting temperature of C-terminal domain (Transition I) of T7 RNAP from 48.3 °C to 37.7 °C (Fig. 4F, Table 1). Destabilization of the C-terminal domain could be a potential factor leading to such a decrease. Presence of guanidine hydrochloride does not change the melting temperature of T7 RNAP C-terminal domain. The melting temperature of N-terminal domain however shows a small increase in presence of both arginine and guanidine hydrochloride (Fig. 4G, Table 1).

4. Discussion

Arginine is employed as an agent to improve the refolding yield of recombinant proteins via suppression of aggregation [8]. It has also been shown to enhance the chaperone like activity of α-crystallin [9]. The hydrophobic surface provided by arginine plays a major role in the interaction of this amino acid with proteins [17]. To the best of our knowledge, this is the first report of the activity enhancement of an enzyme upon addition of arginine. We have shown earlier the increase of T7 RNAP activity by guanidine hydrochloride [15]. The activity enhancement by arginine is higher as compared to that by guanidine hydrochloride. In this report, along with the demonstration of activity enhancement, we

Table 1
Thermodynamic parameters for denaturation of T7 RNAP in presence of different ligands as evaluated from differential scanning calorimetry.

Sample	Transition I T_{mI} (°C)	Transition II T_{mII} (°C)
T7 RNAP	48.3 ± 0.03	53.4 ± 0.46
T7 RNAP + 50 mM GdmCl	48.7 ± 0.03	55.2 ± 0.9
T7 RNAP + 100 mM arginine	37.7 ± 0.03	54.2 ± 0.48

have also attempted to understand the structural basis of such enhancement. We have also compared it with guanidine hydrochloride. There are studies [10] to suggest that guanidine hydrochloride and arginine, both containing guanidinium group, interact differently with proteins. Protein stabilization in presence of low concentration of guanidine hydrochloride has been documented in case of Ribonuclease T1, and SH3 domain of Fyn tyrosine kinase [22,23]. There are also reports that demonstrate the enhancement of activity of proteins such as adenylate kinase in presence of small concentration of guanidine hydrochloride [8,10,24]. On the other hand, there are reports which suggest that arginine destabilizes proteins [25] and it inhibits protein aggregation [10,26].

Increase of hydrodynamic diameter and destabilization of the C-terminal domain of T7 RNAP in presence of arginine might originate from the formation of partially unfolded intermediate of T7 RNAP at 100–150 mM concentration of arginine. Guanidine hydrochloride does not alter the hydrodynamic diameter of T7 RNAP. The N-terminal domain of T7 RNAP shows higher melting temperature in presence of guanidine hydrochloride (Table 1), indicating stabilization of N-terminal domain. This result is further supported from our previous observation of the reduced susceptibility of T7 RNAP proteolysis in presence of 50 mM guanidine hydrochloride [15]. Moreover, the denaturation profile of T7 RNAP treated with 50 mM guanidine hydrochloride shows higher value of excess heat capacity compared to untreated T7 RNAP. Calculation of the entropy of unfolding (obtained by calculation of area under the curve of Cp/T vs. T plot) for T7 RNAP treated with guanidine hydrochloride yields higher value as compared to that for native protein. These results indicate that T7 RNAP treated with 50 mM guanidine hydrochloride becomes conformationally more flexible compared to the native protein. Thus, guanidine hydrochloride modulates the flexibility of the enzyme. This observation validates our previous proposition that the conformational change induced by lower concentration of guanidine hydrochloride facilitates the bending of thumb subdomain around residue 388 [15]. Collectively, these observations support the proposition that arginine and guanidine hydrochloride modulate the structure of T7 RNAP differently. Previous reports have shown that guanidine hydrochloride interacts with protein by its charged guanidinium group [22], and in some instances, it modulates the flexibility of proteins [24,27], whereas arginine interacts with proteins by hydrophobic interaction [17]. Thus the mode of interaction of arginine with protein is, in general, different from that of guanidine hydrochloride at the molecular level. Investigation of the energetics of interaction of arginine/guanidine hydrochloride with T7 RNAP reveals another feature which distinguishes the interaction of arginine with the enzyme from that of guanidine hydrochloride. A distinct dip in the enthalpy curve obtained from the titration of T7 RNAP with arginine demonstrates formation of an energetically stabilized intermediate in the concentration range of 75–150 mM.

It has been shown earlier that ligand induced partial unfolding of protein structure lowers the activation energy barrier [28]. This is reflected in the activity profile of many proteins in presence of classical denaturants [8,15,29]. Enhancement of enzymatic activity of T7 RNAP by arginine might be a consequence of lowering of activation energy barrier via formation of a partially unfolded intermediate. On the other hand, activity enhancement of T7 RNAP by lower concentration of guanidine hydrochloride is probably brought about by altered flexibility of T7 RNAP structure. Finally, it may be conjectured that both of these effects culminate in the stabilization of the ternary complex, T7 RNAP–DNA–RNA, thereby favoring transition from abortive cycling to processive transcription.

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