ELSEVIER

Biophysical Chemistry 103 (2003) 223–237

Biophysical Chemistry

www.elsevier.com/locate/bpc

Inter-subunit recognition and manifestation of segmental mobility in *Escherichia coli* RNA polymerase: a case study with ω–β′ interaction

Pallavi Ghosh[a,b], Chandrashekharan Ramakrishnan[b], Dipankar Chatterji[b,*]

[a]*Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, A.P., India*
[b]*Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, Karnataka, India*

Received 16 July 2002; received in revised form 3 September 2002; accepted 4 September 2002

Abstract

Omega (ω), consisting of 91 amino acids, is the smallest of all the *Escherichia coli* RNA polymerase subunits and is organized into an N-terminal domain of 53 amino acids followed by an unstructured tail in the C-terminal region. Our earlier experiments have shown a chaperone-like function of ω in which it helps to maintain β′ in a correct conformation and recruit it to the $\alpha_2\beta$ subassembly to form a functional core enzyme ($\alpha_2\beta\beta'\omega$). The X-ray structure analysis of *Thermus aquaticus* core RNA polymerase suggests that two regions of ω latch onto the N-terminal and C-terminal ends of the β′-subunit. In the present study we have monitored the conformational changes in β′ as the denatured protein is refolded in the presence and absence of ω using tryptophan fluorescence emission of β′ as well as acrylamide quenching of Trp fluorescence. Results indicate that the presence of stoichiometric amounts of ω is helpful in β′ refolding. We have also monitored the behavior of the C-terminal tail of ω by engineering three cysteine residues at three different sites in ω and subsequently labeling them with a sulphydryl-specific fluorescent probe. Fluorescence anisotropy measurements of the labeled protein indicate that the C-terminal domain of ω is mobile in the free protein and gets restrained in the presence of β′. Calculations on side-chain interactions show that out of the three mutated positions, two have near neighbourhood interactions only with side-chains in the β′ subunit whereas the end of the C-terminal of ω, although it is restrained in the presence of β′, has no interacting partner within a 4-Å radius.

Keywords: β′; ω; *Escherichia coli* RNA polymerase; Subunits; Fluorescence anisotropy; Domain interaction

1. Introduction

The key enzyme for gene expression in both prokaryotes and eukaryotes is DNA-dependent RNA polymerase. In all organisms this enzyme is a large multi-subunit protein, with the subunits being conserved across species. Bacterial core RNA polymerases contain five subunits, alpha (α) beta (β), beta′ (β′), omega (ω) and sigma (σ) [1]; the archaeal and eukaryotic polymerases are much larger and are comprised of 10–12 subunits [2,3]. Some of the subunits have very critical

*Corresponding author. Tel.: +91-80-309-2836; fax: +91-80-360-0535.
E-mail address: dipankar@mbu.iisc.ernet.in (D. Chatterji).

0301-4622/03/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0301-4622(02)00271-5

functions; however, in most cases the presence of all the subunits is required for enzyme activity.

The ω subunit is the smallest of all the RNA polymerase subunits; in *Escherichia coli* this protein has a molecular weight of 10 kDa and is made up of 91 amino acids [4]. ω homologs are present in most bacteria whose genomes have been sequenced and are also present in archaebacteria [5] and eukaryotes such as yeast, drosophila and even humans [6]. Sequence analysis shows the presence of three regions of homology, namely, CR1, CR2 and CR3 [6] suggesting some important conserved function. However, ω-deleted strains of *E. coli* lacking ω are viable and show no discernable phenotype other than a slow growth rate [7]. The ω-deleted RNA polymerase lacking ω is, however, less active than the wild type polymerase [8] and is associated with large amounts of GroEL [9] thus implying that ω might have a chaperone-like function in maintaining the structure of RNA polymerase or any of its particular subunits. The crystal structure analysis of *Thermus aquaticus* RNA polymerase supports this contention [6,10].

We have previously shown that the chaperone function of ω is specific for the β′ subunit, the only subunit of RNA polymerase that is not folded in vivo by GroEL [11]. ω has the ability to prevent β′ aggregation during its refolding in vitro; it binds simultaneously to the N-terminal and C-terminal portions of β′, thereby maintaining it in a correct conformation and recruits it to the $\alpha_2\beta$ subassembly to form a functional $\alpha_2\beta\beta'$ core RNA polymerase [12]. However, the nature of interaction between ω and β′ remains elusive as we do not have any structural information on ω-less RNA polymerase thus limiting us to follow the change in conformation of β′ upon ω binding. In the present work, we have attempted to monitor the conformational changes in β′ as the denatured protein is refolded in the presence and absence of ω. Tryptophan fluorescence emission of β′ as well as acrylamide quenching of Trp fluorescence indicate that β′ refolds into its native conformation in the presence of stoichiometric amounts of ω.

We have previously identified a 53 amino acid N-terminal fragment of ω which retains all the functions of the full-length protein [12]. The C-terminal region from 54 to 91 amino acids seems to be highly unstructured as determined by limited proteolysis. In the present work we have engineered cysteine residues to create specific labeling sites for fluorescent probes and carried out fluorescence spectroscopic analysis to study the behavior of the C-terminal portion of ω upon its association with the β′ subunit. The wild type ω subunit of *E. coli* has no cysteine residues. Our results show that the C-terminal tail of the ω subunit is constrained in the presence of β′.

2. Materials and methods

2.1. Construction of cysteine mutations by site-specific mutagenesis

Site-specific mutagenesis was carried out by the *QuickChange* protocol (Stratagene). Three single cysteine mutants, G57C, R67C and R90C were constructed using this method. The mutations were confirmed by sequencing the DNA (sequencing was carried out at the Department of Biochemistry, IISc, Bangalore) as well as MALDI-TOF mass spectrometry (KRATOS) of the purified proteins.

2.2. Protein purification and characterization

ω Mutants as well as wild type ω were expressed in *E. coli* BL21 (DE3) and purified as previously described [13]. As the buffer system used for purifying wild type ω was devoid of any reducing agent, DL-dithiothreitol (DTT; 1 mM) was now included in all the buffers during purification to minimize thiol oxidation. β′ was expressed and purified as previously described [14].

2.3. Modification of single cysteines with 5-[2-(2-iodoacetamidoethylamino)]-1 naphthalenesulphonic acid

Covalent modification of single cysteine residues were carried out at pH 8.0 in 40 mM Hepes containing 50 mM KCl, 0.1 mM EDTA and 5% glycerol [15]. Protein samples were incubated with 20-fold molar excess of 5-[2-(2-iodoacetamidoethylamino)]-1 naphthalenesulphonic acid (IAEDANS) for 1 h in the dark at 25 °C followed by

a further incubation of 12 h at 4 °C. A 10-fold molar excess of DTT over the molar concentration of IAEDANS was then added to stop the reaction. The unbound probe was removed by extensive dialysis against several changes of the same buffer. The labeled protein was then separated from the unlabeled protein on a Mono Q (Pharmacia) anion-exchange column. The modified protein was checked for its biological activity towards reconstitution of RNA polymerase as well as native conformation by circular dichroism studies.

2.4. Analysis of secondary structure

Far UV CD spectra of ω and its 5.7 kDa fragment were recorded on a JASCO J-715 CD spectropolarimeter. Mean residue ellipticity $[\theta] = (1000\theta m)/LC$ where θ is the measured ellipticity in degrees, m is the mean residue weight in g/dmol, C is the concentration in g/l and L is the path length in cm.

2.5. Assembly of ω into RNA polymerase

ω-less RNA polymerase purified from *rpoZ* null mutants was reconstituted with ω its cysteine mutants, and the labeled derivatives as described by Mukherjee and Chatterji [8]. In all the cases, ω was added at a 10-fold molar excess over RNA polymerase as described earlier [8]. Aliquots removed at different time points were used for a calf thymus DNA as the template in multiple-round general transcription assay [16].

2.6. Binding of β′ and ω

In all binding reactions of ω and β′, β′ (1 mg/ml) was denatured in 8 M urea in a buffer containing 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 10 mM DTT, 10 mM $MgCl_2$, 0.2 M KCl and 20% glycerol for 8 h at 4 °C. ω (equal molar concentration) was added and the reaction was continued for another hour. The proteins were then refolded together by removal of urea by dialyzing against a refolding buffer containing 50 mM Tris–HCl pH 7.8, 0.1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 0.3 M KCl and 20% glycerol.

2.7. Tryptophan fluorescence measurements of β′

0.8 nmol of β′, denatured in 8 M urea was refolded by the removal of urea in the presence and absence of stoichiometric amounts of ω. Fluorescence spectra were measured on a SPEX Fluoromax-3 spectrofluorometer. Tryptophan emission was recorded at 25 °C in the correction mode between 315 and 380 nm using an excitation wavelength of 295 nm.

2.8. Fluorescence quenching experiments

Steady-state fluorescence quenching experiments were performed at 20 °C by titrating the above solutions of free ω mutants, free β′ and ω-bound β′ samples with freshly prepared 1-M solutions of acrylamide (Amersham Pharmacia). The quenching of fluorescence emission at the constant wavelength of maximum emission was calculated with the Stern–Volmer equation [17]:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 and F are the fluorescence intensity in the absence and in the presence of quencher (Q), respectively, K_{SV} is the Stern–Volmer quenching constant determined from the slope of F_0/F as a function of the quencher concentration $[Q]$.

2.9. Fluorescence anisotropy measurements

AEDANS-labeled ω proteins (0.8 nmol) were mixed with increasing amounts of denatured β′ (β′:ω ratios of 0, 0.3, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 4.0 and 5.0). The subunits were then refolded together by removal of urea by dialyzing against the refolding buffer. Steady-state anisotropy measurements were recorded using a SPEX Fluoromax-3 spectrofluorometer equipped with autopolarizers using the standard program supplied by the manufacturers. G-factor corrections were carried out automatically by the machine.

2.10. Analysis of ω–β′ interaction in the T. aquaticus crystal structure

Using the algorithm CLUSTAL W we identified residues Asp 57, Arg 72 and Ala 98 in *T. aquaticus*

ω subunit to correspond to the *E. coli* residues at positions 57, 67 and 90. A small algorithm was written to identify the residues in β′ that fall within a radius of 4 Å of each of these residues in ω using the available crystal structure coordinates of *T. aquaticus* RNA polymerase. Further, we have substituted Asp 57, Arg 72 and Ala 98 to cysteines at each of these positions and then identified the residues in β′ which show interactions with the side-chain of the cysteines within a radius of 4 Å.

3. Results

3.1. Conformational changes in β′

Our earlier studies [12] have shown that β′, which is the largest subunit of RNA polymerase, is highly prone to aggregation during its process of folding. We then showed a chaperone-like function of ω in which ω helps in preventing aggregation of β′ as it renatures into its native state [12]. Here we have studied the conformational changes accompanying β′ refolding using the intrinsic fluorescence of tryptophan. The fluorescence characteristics of tryptophan residues depend strongly on the microenvironment and thus provide a sensitive probe for the conformational state of proteins. In general, when a protein unfolds, it exposes buried tryptophans to the aqueous solvent resulting in the shift of fluorescence emission maxima towards longer wavelength of 355 nm [17,18]. Measurement of intrinsic fluorescent properties of substrate proteins has been used previously to study protein interactions with chaperones such as GroEL [19] and DnaJ [20]. Our study is facilitated by the fact that ω itself lacks tryptophan residues and the emission spectrum is contributed entirely by the tryptophans of β′. As seen in Fig. 1 the fluorescence spectrum of native β′ has a maximum at 334 nm. When β′ is denatured in the presence of 8 M urea, the emission maximum shifts to 340 nm indicating the partial unfolded nature of the protein. It should be mentioned at this point that β′ has several tryptophan residues distributed all over the primary sequence and thus the emission spectrum of β′ reported here is an average value. Thus a shift of 6 nm upon denaturation is of significance here. β′ renatured in the absence of ω has an emission maximum of 337 nm indicating a state intermediate between that of the denatured and the native conformation of β′. However, when refolded in the presence of stoichiometric amounts of ω, the emission maximum is restored to 334 nm which is identical to that of the native protein. These results suggest that β′ can be folded into its native conformation in the presence of ω.

We have further probed the changes in β′ conformation upon refolding using the quenching of tryptophan fluorescence by acrylamide. The exposure of β′ tryptophans to the external solvent as it refolds to its native conformation was determined by monitoring the fluorescence intensity of β′ in the presence of increasing concentrations of acrylamide (0–80 mM). Fig. 2 shows the Stern–Volmer plots of β′ fluorescence quenching by acrylamide as β′ is refolded in the absence and presence of stoichiometric amounts of ω. The Stern–Volmer constants (K_{SV}) which are representative of solvent exposure, are summarized in Table 1. Unfolding of β′ results in a solvent exposure of tryptophan residues as indicated by the largest extent of quenching ($K_{SV}=1.60$). The native state ($K_{SV}=1.0$) as well as β′ refolded in the presence of ω ($K_{SV}=0.98$) show least accessibility to the quencher, whereas β′ renatured in absence of ω shows an intermediate accessibility to acrylamide ($K_{SV}=1.16$). These results corroborate the results obtained in the previous section from tryptophan fluorescence and further indicate that β′ can achieve its native folded state only in the presence of ω. It should be mentioned that the Stern–Volmer plot shown here is an average of two independent experiments at each point which yielded very close values and thus we did not calculate standard-deviation or other statistical parameters. Computer drawn best fit lines at times did not pass through 1, as the case should be and had small variations (0.95–1.05). Thus, our interpretation of K_{sv} have small errors attributed to this variations which do not alter the conclusion drawn here.

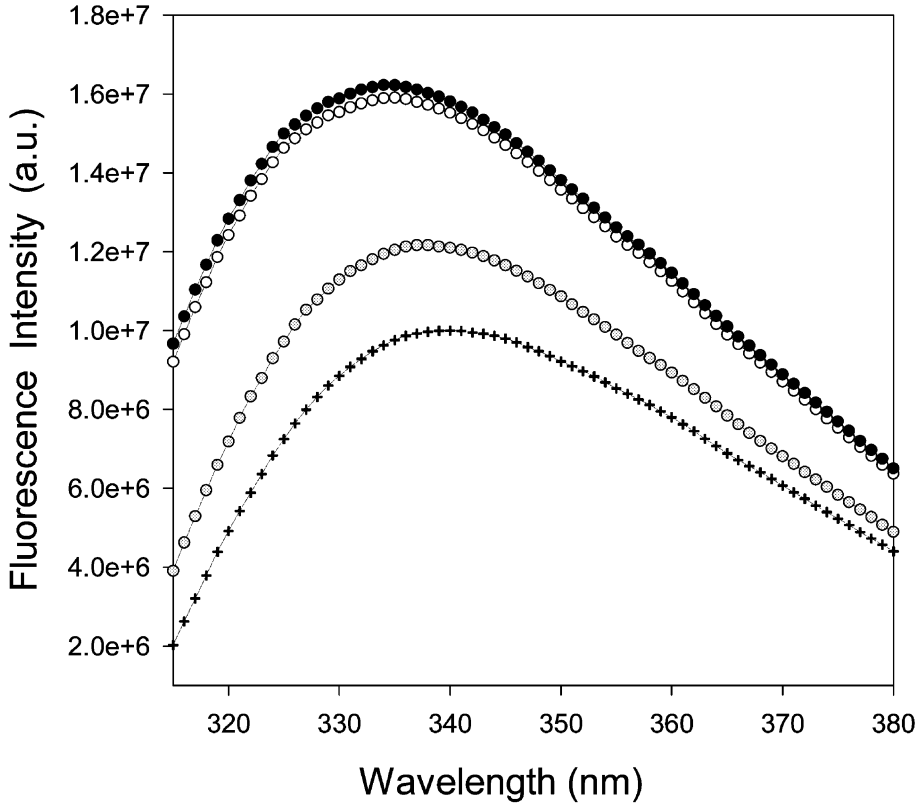

Fig. 1. Fluorescence emission spectra of β′: 0.8 nmol of β′ was denatured in 8 M urea, ω was added and the proteins were then refolded together by removal of urea. Tryptophan emission spectra of native β′ (open circles), denatured β′ (cross marks), and β′ renatured in the absence (grey filled circles) and presence (black filled circles) of ω.

3.2. Construction and characterization of single cysteine mutations

The crystal structure of ω associated in the *T. aquaticus* RNA polymerase shows the presence of secondary structure elements from 3 to 65 amino acids spanning the regions of sequence conservation CR1 and CR3 [6]. There are two α-helices in the CR1 region (2–34 amino acids of *E. coli*), whereas the first half CR3 (46–65 amino acids in *E. coli*) corresponds to the third helix and the second half to a β-strand [6]. However, our previous studies on the structure of free ω using limited proteolytic digestion with the V8 protease, revealed that ω is structured into a 53 amino acid domain in the N-terminal portion of the protein, whereas the remaining region from 54 to 91 amino acids is disordered as it is rapidly cleaved by the protease [12]. Thus, the region 50–65 amino acids which is unstructured in the free protein seems to become structured when associated in the polymerase. In the present study we have now employed fluorescent methods to monitor conformational alterations and the behavior of this C-terminal tail upon complex formation with β′. This method involves the measurement of fluorescent signals originating from a probe attached at different positions of the tail. In order to be able to label ω with a specific fluorescent probe we have constructed single Cys mutant ω derivatives bearing cysteines at different locations with respect to the N-terminal domain (Fig. 3a). These are: (i)

immediately after the N-terminal domain, G57C (ii) at the terminal residue of the 'tail', R90C and (iii) at a position in the middle of the tail, R67C. The sites of mutation were chosen so as to preserve the activities of the derivatives by mutating only the non-conserved residues. All the mutants were overexpressed, purified and used in the reconstitution assay of the ω-less enzyme. The efficiency of reconstitution remained unaltered for all the mutants (Fig. 3b).

3.3. Labeling of Cys mutants with IAEDANS and characterization of the labeled proteins

All the three mutants were modified with the cysteine specific probe IAEDANS. All labeled proteins were then separated from their unlabeled counterparts on an anion exchange resin (MonoQ, Pharmacia). The stoichiometry of labeling was estimated to range from 0.9 to 1.0 as determined by the ratio of concentrations of ω·[$\varepsilon_{210} = 2.5\times10^{-5}$/M] (this work) and IAEDANS [$\varepsilon_{336} = 6.1\times10^{-3}$/M] [21] implying that IAEDANS was incorporated specifically at the cysteine residues. The absorbance and emission spectra of the AEDANS-labeled ω subunits are shown in Fig. 4a and b. AEDANS conjugated to ω has an absorption λ_{max} of 336 nm and an emission λ_{max} of 495 nm indicating that neither the λ_{max} of absorption nor the λ_{max} of emission of IAEDANS changes upon

Table 1
Stern–Volmer (K_{SV}) quenching constants for β′ in different stages of folding

	K_{SV} (M^{-1})
Native β′	1.0
Denatured β′	1.6
β′ renatured in the absence of ω	1.16
β′ renatured in the presence of ω	0.98

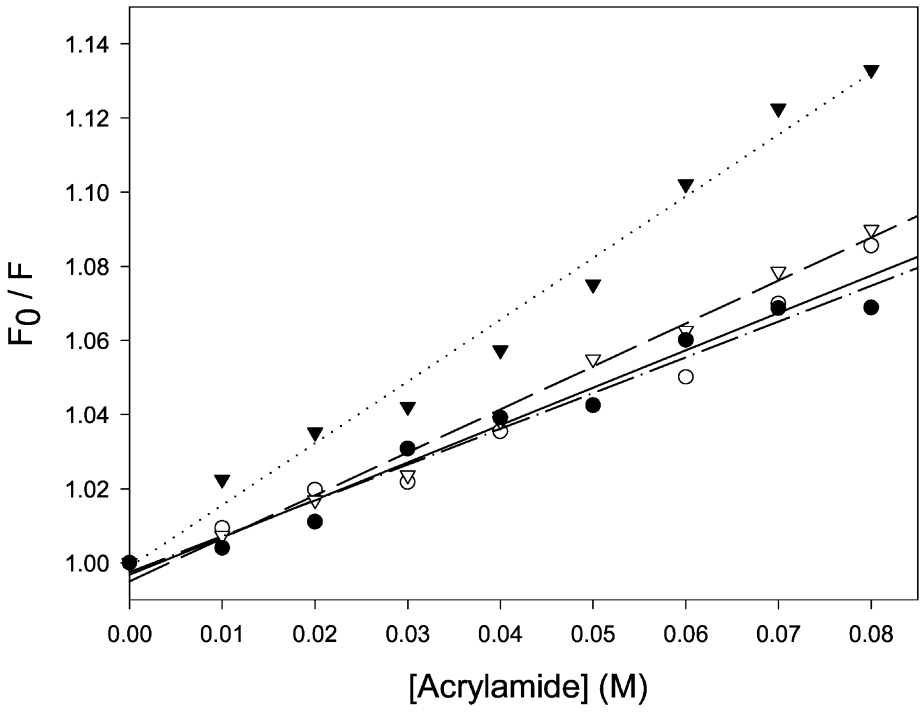

Fig. 2. Stern–Volmer plot for the quenching of β′ tryptophan fluorescence by acrylamide: *F* is the measured fluorescence intensity in the presence of the indicated concentration of acrylamide and F_0 is the initial fluorescence intensity in the absence of acrylamide. Denatured β′ (…filled triangles…), native β′ (_ _ open circles_ _ _), β′ denatured in the absence (----open triangels----) and presence (––open squars––) of ω. Tryptophan emission was recorded between 315 and 380 nm using an excitation wavelength of 295 nm.

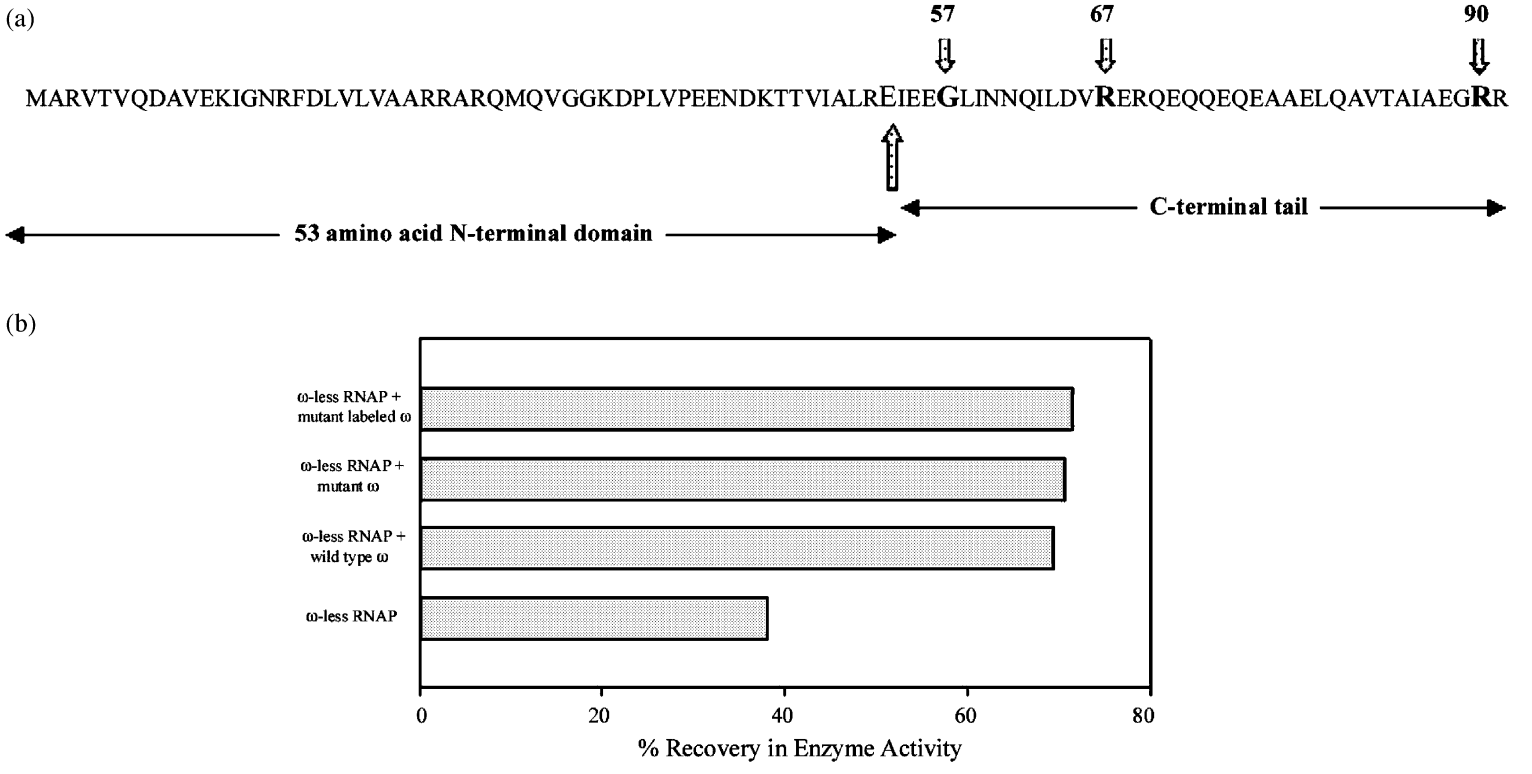

Fig. 3. Construction of cysteine mutations and their characterization. (a) Construction of cysteine mutations at indicated positions on the tail. (b) Reconstitution of ω-less RNA polymerase in the absence and presence of wild type ω, cysteine mutants of ω and AEDANS labeled ω. All mutants as well as the labeled mutants reconstitute ω-less RNAP with the same efficiency and therefore only a representative is shown in either case.

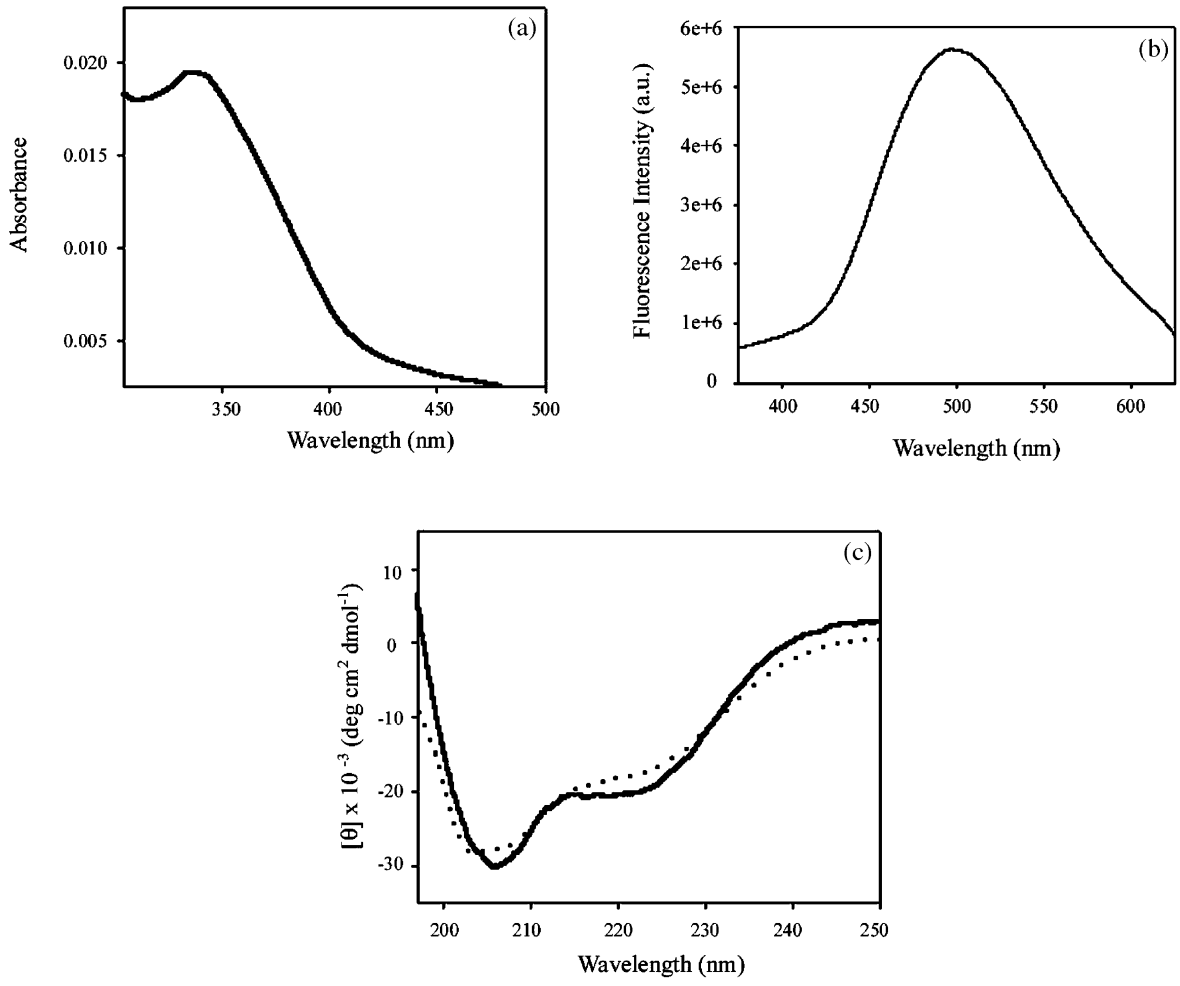

Fig. 4. Characterization of AEDANS labeled species. (a) Absorbance spectra of AEDANS-labeled ω. (b) Emission spectra of AEDANS-labeled ω. (c) Far UV CD spectrum of wild type ω (solid line) and a representative ω cysteine mutant (dotted line).

conjugation with ω. The structure of all the three mutants were unaltered after labeling as determined by CD studies (Fig. 4c). Moreover, their ability to reconstitute the activity of an ω-less RNA polymerase was also found to be unaltered (Fig. 3b).

3.4. Probing the mobility of AEDANS at different regions of ω upon interaction with β′

Following the labeling of mutants at their respective positions with AEDANS, we have then probed the mobility of AEDANS at different regions of ω upon interaction with β′. Measurement of fluorescence anisotropy has been widely used to study protein–protein [22,23] and protein–DNA interactions [22,24]. The behavior of the probe at a particular residue can be considered to be representative of that region of the protein. This experiment is predicated on the difference in rotation rates (and consequently fluorescence anisotropies) of different regions of the C-terminal tail of ω, free in solution and in complex with β′. According to the principle of fluorescence polarization [17] anisotropy values for free ω are expected to be low in regions of the protein that are in

Table 2
Absolute anisotropies of AEDANS at different locations in free ω

AEDANS positions	Anisotropy $= I_{\parallel} - I_{\perp} / I_{\parallel} + 2I_{\perp}$
ωC57	0.191 ± 0.002
ωC67	0.096 ± 0.003
ωC90	0.073 ± 0.003

rapid motion. If a region becomes constrained upon association with β′, it would then show an increase in anisotropy. Thus the difference in anisotropy between the free and the β′-bound forms can be expected to shed light on the conformational status of the C-terminal region of ω in solution and in association with β′.

Fluorescence anisotropy for AEDANS at C57, C67 and C90 positions of ω bound to β′ or free in solution was measured at 25 °C. Measurements were carried out at the emission maximum of AEDANS (495 nm) in the correction mode. Data was expressed as $(A - A_0)/A_0$ where A denotes fluorescence anisotropy in the presence of indicated concentrations of β′ and A_0 denotes the fluorescence anisotropy in the absence of β′. It was observed that the absolute anisotropy value of AEDANS at position C90 is the least followed by C67 and being the highest for C57 (Table 2). This suggested that in the free protein the distal end of the tail is in rapid motion whereas the proximal end (C57) is more rigid as a consequence of being near the N-terminal domain region. The middle portion of the tail (C67) is nearly as mobile as the terminal region. Fig. 5 shows that addition of β′ to ω labeled at positions C67 and C90 results in a large increase in fluorescence anisotropy, which saturates at a β′:ω ratio of between 1 and 1.2. Anisotropy differences are slightly higher in the case of the distal region of the tail (residue 90) as compared to the middle of the tail (residue 67) implying that the terminal region of the tail gets maximally constrained upon association with β′; the mid region is also constrained in its movements upon interacting with β′, though to a slightly lesser

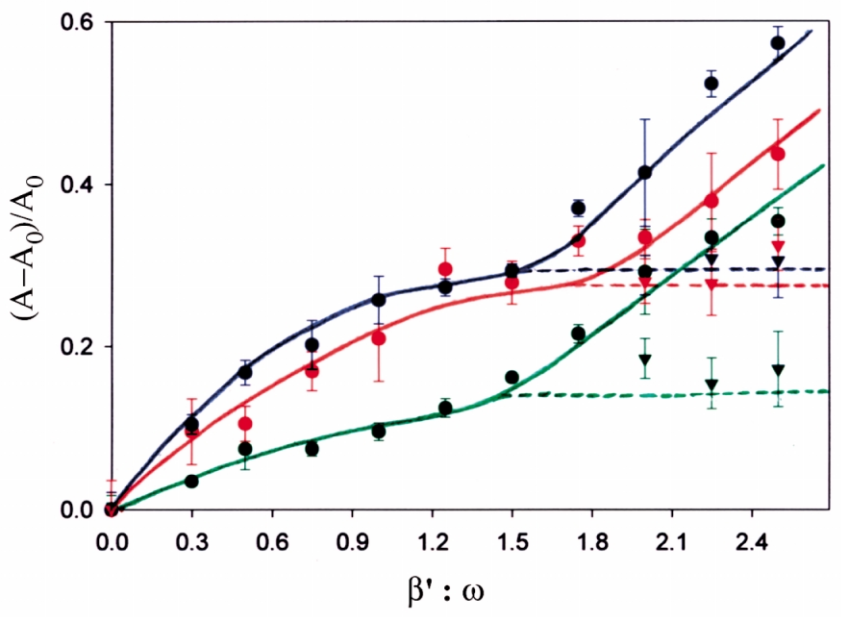

Fig. 5. Fluorescence polarization analysis of ω–β′ interaction: Mutant AEDANS-labeled ω (C57 (green circles), C67 (red circles) and C90 (blue circles)) proteins (0.8 nmol) were mixed with increasing amounts of denatured β′ (β′:ω ratios of 0, 0.3, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, 2.75, 3.0, 4.0, and 5.0) and refolded together. Anisotropy measurements were recorded using a SPEX Fluoromax-3 spectrofluorometer equipped with autopolarizers. At β′:ω ratios of 2, 2.25 and 2.5, ω (C57 (green triangles), C67 (red triangles) and C90 (blue triangles)) was added to restore stoichiometric concentrations between β′ and ω (dotted lines).

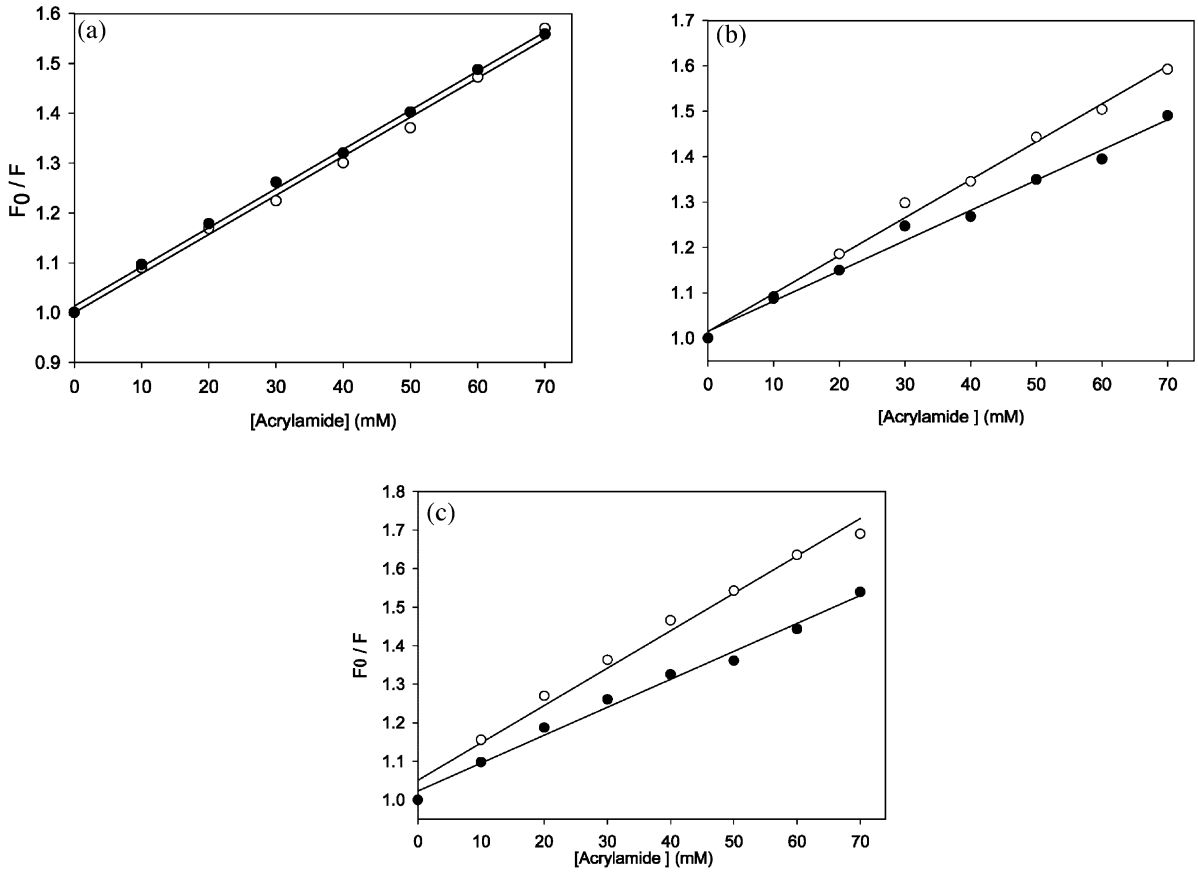

Fig. 6. Stern–Volmer plots for the quenching of AEDANS fluorescence by acrylamide: Stern–Volmer plots of F_0/F versus quencher concentration, for quenching of the AEDANS fluorescence by acrylamide when AEDANS is attached to the C57 (a); C67 (b) and C90 (c) positions of ω. Accessibility to the quencher was studied in the presence (●) and absence (○) of stoichiometric amounts of β′.

degree than the tail end. There is however not much change in anisotropy at the C57 position as the residue is very close to the N-terminal domain and does not undergo much change in mobility upon interaction with β′. This is followed by a sudden non-specific increase in anisotropy upon increasing the β′:ω ratio beyond 2 (Fig. 5). As we have shown earlier, β′ has a tendency to aggregate and this can be inhibited only in the presence of ω. Therefore, as the amount of β′ increased, aggregates could be formed, which on association with ω could show large anisotropy values. We have therefore added external ω to stoichiometric concentrations. This resulted in disruption of these aggregates and a concomitant restoration of anisotropy values to saturable levels (Fig. 5).

3.5. Probing the accessibility of AEDANS at different regions of ω upon interaction with β′

We have studied the behavior of different portions of the C-terminal tail of ω upon association with β′ further by probing the accessibility of AEDANS (conjugated at different locations in ω) to a quencher. The fluorescence of extrinsic probes has been shown to be susceptible to various kinds of quenchers. Since acrylamide has been previously used to quench the fluorescence of dansyl probes

Table 3
Stern–Volmer (K_{SV}) quenching constants for free ω and in association with β′

Proteins	K_{SV} (M^{-1})
Free C57 ω	7.84
C57 ω+β′	7.85
Free C67 ω	8.37
C67 ω+β′	6.66
Free C90 ω	9.71
C90 ω+β′	7.25

[25], we have used acrylamide to probe the accessibility of AEDANS at different locations in ω upon its interaction with β′ (Fig. 6). The Stern–Volmer constants (Table 3) which are the slopes of F_0/F as a function of quencher concentration, provide a relative measure of the degree of accessibility of the fluorescent group to the quencher. In this experiment we have assumed that the fluorescence lifetime of AEDANS is not significantly different in the free and the β′ bound forms of ω as the fluorescence intensity of AEDANS does not change significantly upon β′ binding (Fig. 7). The K_{SV} values in the free proteins is the least in the case of AEDANS-labeled at C57, followed by C67 and is the highest in the case of C90 indicating that the fluorescence of AEDANS was most efficiently quenched when conjugated at positions C90, followed by C67 and being the least in the case of C57. This suggests that the C90 residue is the most solvent accessible region whereas C57 being close to the domain is the least accessible. Upon association of these mutants with β′ the fluorescence of AEDANS was less efficiently quenched at C67 and C90 positions suggesting that these residues became protected and thereby became less accessible to acrylamide upon binding to β′. There was however not much change in the accessibility of AEDANS at C57 (which is immediately next to the N-terminal domain) upon association with β′.

The X-ray structure of *T. aquaticus* core RNA polymerase has been solved at 3.3-Å resolution [10]. Residues Asp 57, Arg 72 and Ala 98 in *T. aquaticus* ω subunit were identified to correspond

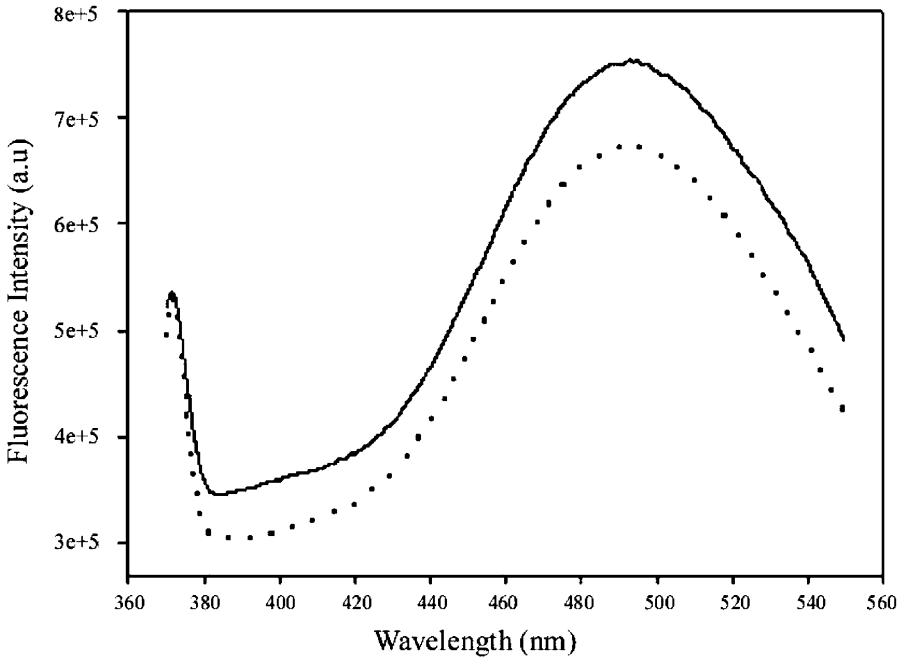

Fig. 7. Emission spectrum of AEDANS conjugated to ω: The emission spectrum of AEDANS was recorded in free ω (dotted lines) as well as in association with β′ (solid line).

Table 4
Residues in β′ that fall within a radius of 4 Å of residues in wild type ω

Residue in *E. coli* chain *E*(ω)	Residue in *T. aquaticus* chain *E*(ω)	Interacting residue in *T. aquaticus* chain *D*(β′)
Gly 57	Asp 57	His 696
Arg 67	Arg 72	Asp 1489, Gln 1490
Arg 90	Ala 98	None

to the *E. coli* residues at positions 57, 67 and 90. Using the available crystal structure co-ordinates we have then determined the residues in β′ that fall within a radius of 4 Å of each of these residues in ω (Table 4 and Fig. 8A–C) as a distance of 4 Å is considered to be optimum for the side-chains of two residues to interact. No other residues show any side-chain interactions with ω at this distance. A small algorithm was written to facilitate this procedure. Further, we have substituted Asp 57, Arg 72 and Ala 98 to cysteines at each of these positions and then identified the residues in β′ which show interactions with the side-chain of the cysteines within a radius of 4 Å. We also noticed that these interactions remain unaltered after cysteine substitution. On comparing our results with the available information from the crystal structure we find that residue ω 72 (67 in *E. coli*) shows the maximum interactions with β′ residues thereby explaining the decreased accessibility to acrylamide and an increase in anisotropy. Interestingly this region forms an antiparallel β sheet with residues 1483–1487 of the β′ subunit in a manner that 'clamps' it in a crevice formed by CR1 and the first half of CR3 of ω [6]. Residue 98 (90 in *E. coli*), however, does not show any interaction with β′ residues at a distance of 4 Å. Nonetheless, this region also shows an increase in anisotropy values as compared to the free protein perhaps due to the increase in mass for probe attachment. It is tempting to suggest that this restricted mobility may be a result of the effects of β′ binding at the clamp region (represented by the amino acid at position 67) of the tail being transduced to the distal tail portions. However, it may also be a consequence of the presence of the bulky AEDANS moiety. We have determined the side-chain interactions between ω and β′ at a distance of 10 Å to account for the presence of AEDANS. At this cut-off, the residue at position 90 (98 in *T. aquaticus*) also begins to show interactions with β′ (data not shown).

4. Discussion

Objective of this manuscript was to study the nature of interaction between the ω and β′ subunits of *E. coli* RNA polymerase which make them such a specific partner during the assembly process of the enzyme. We have studied the changes in both proteins as they interact with each other. In our earlier study we have shown that β′ tends to aggregate when refolded in vitro and that this aggregation can be prevented by the addition of the ω subunit implying that ω can bind β′ and help in its process of refolding [12]. We have extended this study here further by directly monitoring the conformation of β′ as it refolds from its denatured state in the absence and presence of ω. Further, we have also studied the accessibility of tryptophans, during β′ renaturation, to acrylamide which acts as a quencher of fluorescence. Results are presented in Figs. 1 and 2. The emission maximum of tryptophan shows a shift from 334 to 340 nm as it unfolds from its native to a denatured state. Upon refolding in the presence of stoichiometric amounts of ω, β′ regains its conformation to that of the native state as characterized by a shift in tryptophan fluorescence to 334 nm. When β′ is unfolded in the absence of ω it attains a conformation intermediate between the native and the denatured state as characterized by a tryptophan emission maximum of 337 nm. The accessibility of tryptophan to acrylamide also showed a similar trend with tryptophans being most accessible in the denatured form; the native and ω-induced renatured forms of ω showed the least accessibility to acrylamide whereas β′ renatured in the absence of ω showed an intermediate exposure of tryptophans. These results together show that β′ can be folded into its native conformation only in the presence of ω.

The second aspect of this work involves the study of the C-terminal tail of ω which, from our

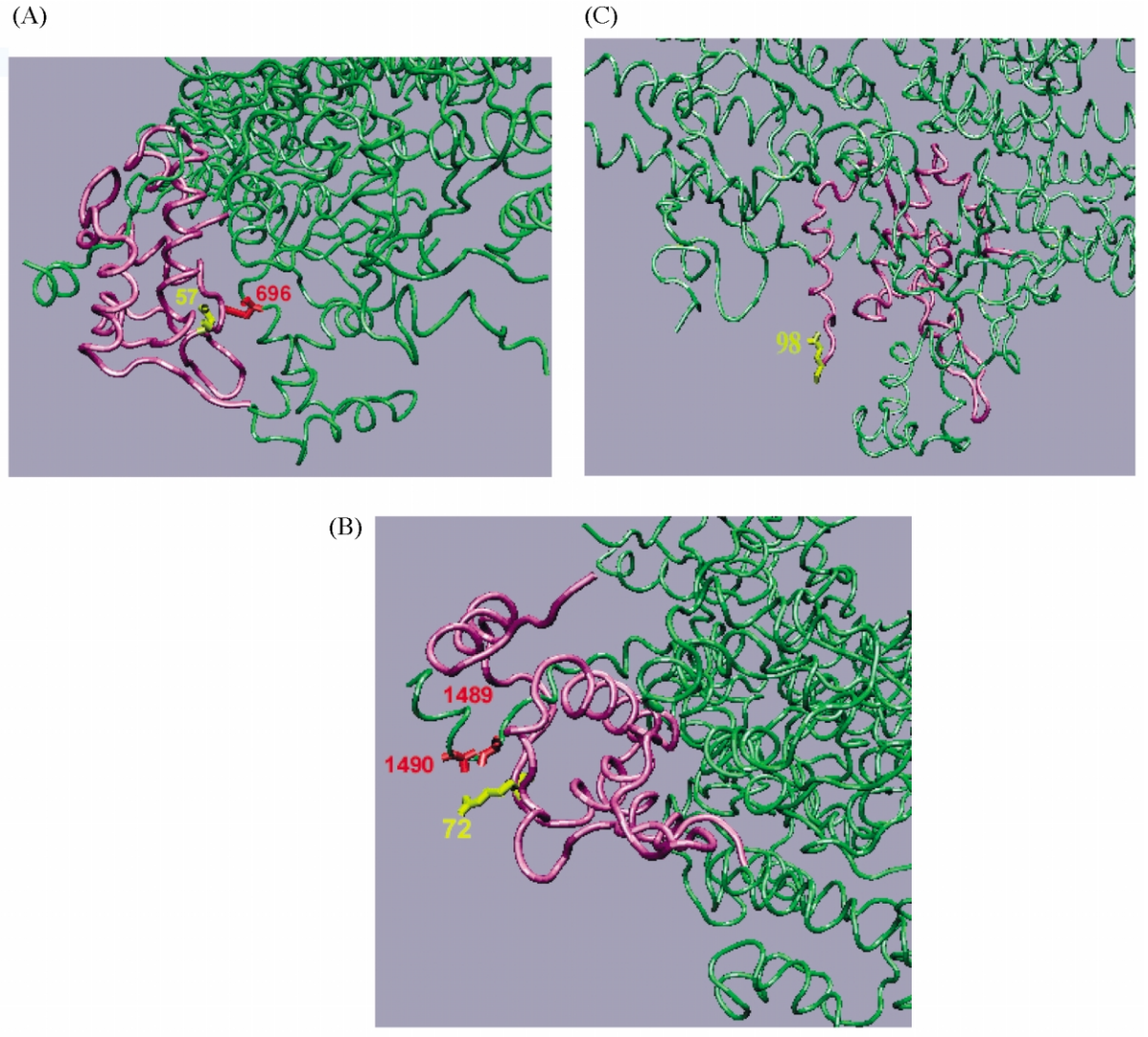

Fig. 8. ω–β′ interactions in the *T. aquaticus* RNA polymerase: Residues Asp 57, Arg 72 and Ala 98 (shown in yellow) in *T. aquaticus* ω subunit were identified to correspond to the *E. coli* residues at positions 57, 67 and 90. Using the available crystal structure co-ordinates, residues Asp 57, Arg 72 and Ala 98 were substituted to cysteines at each of these positions. The residues in β′ which show interactions with the side-chain of the cysteines within a radius of 4 Å are shown in red.

earlier work [12], appears to be an unstructured stretch of 38 amino acids. To this end, residues at different regions of the tail were changed to a cysteine to enable labeling with a cysteine-specific probe, IAEDANS. We have then probed the mobility of AEDANS at different regions of ω upon interaction with β′ using the technique of fluorescence anisotropy. The anisotropy values of AEDANS at position C90 are the smallest followed by C67 and being the highest for C57. This indicated that in the free protein the distal end of the tail is in rapid motion whereas the proximal end (C57) is more rigid as a consequence of being near the N-terminal domain region. Difference in anisotropy $(A-A_0)$ between the β′ complexed protein and the free protein shows that the tail

becomes highly constrained upon association with β′. This is further demonstrated by the results of acrylamide quenching of AEDANS fluorescence; the solvent accessibility remains unchanged in the C57 position upon β′ binding whereas regions in the middle and at the end of the tail become more protected upon binding β′ as revealed by a decreased accessibility to acrylamide.

The above results show that the C-terminal tail, which is in random motion in the free protein, upon association with the β′ subunit, is restricted in its mobility due to the interactions it makes with β′. An analysis of the β′–ω interface in the crystal structure of *T. aquaticus* core RNAP [6] also confirmed the involvement of the residues in the tail portion to interact with β′, although the majority of interactions are contributed by the ω N-terminal domain. This is also in agreement with our previous work where we have shown that although the C-terminal region is not involved in ω function, its deletion reduces the overall efficiency of the ω N-terminal domain [12]. From these results we can conclude that the C-terminal tail contributes to the binding to RNA polymerase, specifically the β′ subunit. Thereby, deletion of the tail reduces the efficiency of ω function by decreasing the binding to β′.

An interesting aspect is the specificity of ω for the β′ subunit of RNA polymerase. This specificity is true not only in *E. coli* but has also been shown in *T. aquaticus* [10] and *S. cerevisiae* [6] (ω homolog RPB 6 interacts with β′ homolog RPB 1). Our side-chain interaction studies with three different positions in ω show only the β′ subunit as a partner within a radius of 4 Å indicating major functional implications for such interactions. The mobility of the C-terminal tail of ω helps it to latch onto the β′ subunit. Sequence specificity for such interactions could be utilized further to modulate the assembly process of this important enzyme.

Acknowledgments

One of us (PG) acknowledges Senior Research Fellowship from CSIR, India. The work was partly supported by Department of Biotechnology, Govt. of India.

References

[1] R.R. Burgess, Separation and characterisation of the subunits of RNA polymerase, J. Biol. Chem. 244 (1969) 6168–6176.
[2] J. Archambault, J.D. Friesen. Principles that govern the folding of protein chains. 57 (1993) 703–724.
[3] S.D. Bell, S.P. Jackson. Mechanism and regulation of transcription in archaea. 4 (2001) 208–213.
[4] D.R. Gentry, R.R. Burgess, *rpoZ* encoding the omega subunit of *E. coli* RNA polymerase is in the same operon as *spoT*, J. Bacteriol. 171 (1989) 1271–1277.
[5] C.P. Magill, S.P. Jackson, S.D. Bell, Identification of a conserved archaeal RNA polymerase subunit contacted by the basal transcription factor TFIIB, J. Biol. Chem. 276 (2001) 46693–46696.
[6] L. Minakhin, S. Bhagat, A. Brunning, et al., Bacterial RNA polymerase subunit omega and eukaryotic RNA polymerase subunit RPB6 are sequence, structural, and functional homologs and promote RNA polymerase assembly, Proc. Natl. Acad. Sci. USA 98 (2001) 892–897.
[7] D. Gentry, H. Xiao, R. Burgess, M. Cashel, The omega subunit of *Escherichia coli* K-12 RNA polymerase is not required for stringent RNA control in vivo, J. Bacteriol. 173 (1991) 3901–3903.
[8] K. Mukherjee, D. Chatterji, Studies on the ω subunit of *E. coli* RNA polymerase: its role in the recovery of denatured enzyme activity, Eur. J. Biochem. 247 (1997) 884–889.
[9] K. Mukherjee, H. Nagai, N. Shimamoto, D. Chatterji, GroEL is involved in the activation of *E. coli* RNA polymerase devoid of the ω subunit in vivo, Eur. J. Biochem. 266 (1999) 228–235.
[10] G. Zhang, E.A. Campbell, L. Minakhin, C. Richter, K. Severinov, S.A. Darst, Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 Å resolution, Cell 98 (1999) 811–824.
[11] W.A. Houry, D. Frishman, C. Eckerskorn, F. Lottspeich, F.U. Hartl, Identification of in vivo substrates of the chaperonin GroEL, Nature 402 (1999) 147–154.
[12] P. Ghosh, A. Ishihama, D. Chatterji, *Escherichia coli* RNA polymerase subunit omega and its N-terminal domain bind full-length β′ to facilitate incorporation into the $\alpha_2\beta$ subassembly, Eur. J. Biochem. 268 (2001) 4621–4627.
[13] D.R. Gentry, R.R. Burgess, Overproduction and purification of the ω subunit of *E. coli* RNA polymerase, Protein Expression Purification 1 (1990) 81–86.
[14] K. Igarashi, A. Ishihama, Bipartite functional map of *E. coli* RNA polymerase alpha subunit: involvement of C-

terminal region in transcription activation by CAMP–CRP, Cell 65 (1991) 1015–1022.
[15] A. Raghavan, D. Chatterji, Guanosine tetraphosphate-induced dissociation of open complexes at the *Escherichia coli* ribosomal protein promoters rplJ and rpsA P1: nanosecond depolarization spectroscopic studies, Biophys. Chem. 75 (1998) 21–32.
[16] P.A. Lowe, D.A. Hager, R.R. Burgess, Purification and properties of the σ subunit of *E. coli* DNA dependent RNA polymerase, Biochemistry 18 (1979) 1344–1352.
[17] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Publishing Corporation, New York, 1983.
[18] M.R. Eftink, Fluorescence techniques for studying protein structure, Methods Biochem. Anal. 35 (1991) 127–205.
[19] M.K. Hayer-Hartl, J.J. Ewbank, T.E. Creighton, F.U. Hartl, Conformational specificity of the chaperonin GroEL for the compact folding intermediates of alpha-lactalbumin, EMBO J. 13 (1994) 3192–3202.
[20] T. Langer, C. Lu, H. Echols, J. Flanagan, M.K. Hayer, F.U. Hartl, Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding, Nature 356 (1992) 683–689.
[21] E.N. Hudson, G. Weber, Synthesis and characterization of two fluorescent sulfhydryl reagents, Biochemistry 12 (1973) 4154–4161.
[22] T. Heyduk, Y. Ma, H. Tang, R.H. Ebright, Fluorescence anisotropy: rapid, quantitative assay for protein–DNA and protein–protein interaction, Methods Enzymol. 274 (1996) 492–503.
[23] T. Heyduk, J.C. Lee, Y.W. Ebright, E.E. Blatter, Y. Zhou, R.H. Ebright, CAP interacts with RNA polymerase in solution in the absence of promoter DNA, Nature 364 (1993) 548–549.
[24] N.R. Ellerton, I. Isenberg, Fluorescence polarization study of DNA–proflavine complexes, Biopolymers 8 (1969) 767–786.
[25] C. Weitzman, T.G. Consler, H.R. Kaback, Fluorescence of native single-Trp mutants in the lactose permease from *Escherichia coli*: structural properties and evidence for a substrate-induced conformational change, Protein Sci. 4 (1995) 2310–2318.