

## *Escherichia coli* RNA polymerase subunit $\omega$ and its N-terminal domain bind full-length $\beta'$ to facilitate incorporation into the $\alpha_2\beta$ subassembly

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The  $\omega$  subunit of *Escherichia coli* RNA polymerase, consisting of 90 amino acids, is present in stoichiometric amounts per molecule of core RNA polymerase ( $\alpha_2\beta\beta'$ ). The presence of  $\omega$  is necessary to restore denatured RNA polymerase *in vitro* to its fully functional form, and, in an  $\omega$ -less strain of *E. coli*, GroEL appears to substitute for  $\omega$  in the maturation of RNA polymerase. The X-ray structure of *Thermus aquaticus* core RNA polymerase suggests that two regions of  $\omega$  latch on to  $\beta'$  at its N-terminus and C-terminus. We show here that  $\omega$  binds only the intact  $\beta'$  subunit and not the  $\beta'$  N-terminal domain or  $\beta'$  C-terminal domain, implying that  $\omega$  binding requires both these regions of  $\beta'$ . We further show that  $\omega$  can

prevent the aggregation of  $\beta'$  during its renaturation *in vitro* and that a V8-protease-resistant 52-amino-acid-long N-terminal domain of  $\omega$  is sufficient for binding and renaturation of  $\beta'$ . CD and functional assays show that this N-terminal fragment retains the structure of native  $\omega$  and is able to enhance the reconstitution of core RNA polymerase. Reconstitution of core RNA polymerase from its individual subunits proceeds according to the steps  $\alpha + \alpha \rightarrow \alpha_2 + \beta \rightarrow \alpha_2\beta + \beta' \rightarrow \alpha_2\beta\beta'$ . It is shown here that  $\omega$  participates during the last stage of enzyme assembly when  $\beta'$  associates with the  $\alpha_2\beta$  subassembly.

**Keywords:**  $\beta'$ ;  $\omega$ ; interaction; renaturation; assembly.

The DNA-dependent RNA polymerase is a large multi-subunit enzyme which carries out one of the most important functions of transcribing DNA sequences to RNA within any living organism. The *Escherichia coli* RNA polymerase, which is one of the best-studied polymerases, consists of the  $\alpha$ ,  $\beta$ ,  $\beta'$  and  $\omega$  subunits [1]. The core enzyme ( $\alpha_2\beta\beta'\omega$ ), when associated with one of the multiple species of  $\sigma$  factors initiates transcription at specific promoter sites [2]. The  $\omega$  subunit, encoded by the *rpoZ* gene and consisting of 90 amino acids (molecular mass 10 105 Da), was proposed to be an integral part of the core enzyme several years ago [1]. Since then, however, it has been ignored with respect to its function, because *rpoZ* null mutants of *E. coli* show no characteristic phenotype except slow growth, which has been argued to be due to the polar effect on the nearby *spoT* [3]. We recently found that  $\omega$  is required in the restoration of denatured core RNA polymerase to its functionally active form [4]. Further, we showed that an enzyme purified from an  $\omega$ -less *E. coli* strain recruits large amounts of GroEL [5]; removal of GroEL results in a completely inactive core RNA polymerase which even lacks the ability to associate with  $\sigma^{70}$  [6].

The  $\omega$  subunit is associated at the surface of the holoenzyme and is accessible for interaction with transcriptional

activators [7]. Furthermore, it has been shown that the N-terminal portion of  $\omega$  interacts with polymerase, as fusion of another protein to the N-terminus of  $\omega$  prevents its association with RNA polymerase. In this study, we identified a 52-amino-acid domain in the N-terminal portion (NTD) of  $\omega$  by proteolytic digestion and analysed its structural and functional properties in relation to the wild-type protein.

Assembly of *E. coli* RNA polymerase proceeds via the pathway  $\alpha + \alpha \rightarrow \alpha_2 + \beta \rightarrow \alpha_2\beta + \beta' \rightarrow \alpha_2\beta\beta'$  (premature core)  $\rightarrow E_{\alpha_2\beta\beta'}$  (active enzyme) [8]. The premature core is transcriptionally inactive and requires activation to its mature form, which involves the correct rearrangement of the subunits, primarily the  $\beta'$  subunit, for the RNA polymerase activity to be revealed. The factors responsible for polymerase maturation *in vivo* are largely unknown, but the  $\sigma$  factor and DNA are known to play an enhancing role *in vitro*. Cross-linking experiments reveal that  $\omega$  has a high binding affinity for the  $\beta'$  subunit [9]. Interestingly, the  $\beta'$  subunit of RNA polymerase is the only subunit that does not associate with GroEL *in vivo* [10]. The X-ray structure of *Thermus aquaticus* core RNA polymerase determined at 3.3 Å resolution [11] reveals the presence of one molecule of  $\omega$  per molecule of RNA polymerase. Subsequent analysis of the  $\omega$ - $\beta'$  interface by Minakhin *et al.* [12] led them to propose that two conserved structural domains of  $\omega$  bind conserved regions D and G of  $\beta'$  to its C-terminal tail in a manner that reduces the configurational entropy of  $\beta'$  and facilitates its interaction with the  $\alpha_2\beta$  subassembly. The results presented here show that  $\omega$  simultaneously binds the N-terminal and C-terminal regions of  $\beta'$  and also prevents the aggregation of  $\beta'$  during its renaturation *in vitro*. We also show that  $\omega$  is associated

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Abbreviations: NTD, N-terminal domain; CTD, C-terminal domain.  
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in the last step of polymerase assembly during the incorporation of  $\beta'$  in the  $\alpha_2\beta$  subassembly. These results together indicate that  $\omega$  is required to hold  $\beta'$  in a nonaggregated form and to recruit  $\beta'$  into the  $\alpha_2\beta$  subassembly.

## MATERIALS AND METHODS

### Plasmids

The  $\omega$  gene was subcloned from pE3C-2 [13] into the *Bam*HI–*Sal*I segment of pET21b (Novagen). For the cloning of the NTD, the DNA corresponding to the NTD was PCR amplified and cloned between the *Nde*I and *Xho*I site of pET21b. Plasmids used for expression of  $\alpha$ ,  $\beta'$ ,  $\beta$ ,  $\beta'$ -NTD(1–842)-His<sub>6</sub> and  $\beta'$ -CTD(842–1407)-His<sub>6</sub> were pGEMAX185 [14], pRW308 [15], pGETB, pGETC (1–842) and pGETC (842–1407) [16], respectively.

### Protein purification

Full-length  $\omega$  was purified as described by Gentry & Burgess [13]. For the purification of  $\omega$ -NTD, the insoluble fraction was solubilized in 0.1 M NaH<sub>2</sub>PO<sub>4</sub> and 0.01 M Tris/HCl, pH 8.0, containing 8 M urea. The cleared lysate was then applied to a Ni/nitrilotriacetic acid/agarose matrix (Qiagen) equilibrated with the same buffer. The column was washed with the above buffer of pH 6.3. Proteins were eluted by changing the pH to 4.5. The  $\alpha$ ,  $\beta$ ,  $\beta'$  [14], and His<sub>6</sub>-tagged  $\beta'$  fragments [16] were purified as previously

described.  $\omega$ -less RNA polymerase was purified from an *rpoZ* null mutant strain CF2790 as previously described [4].

### Limited digestion with V8 protease

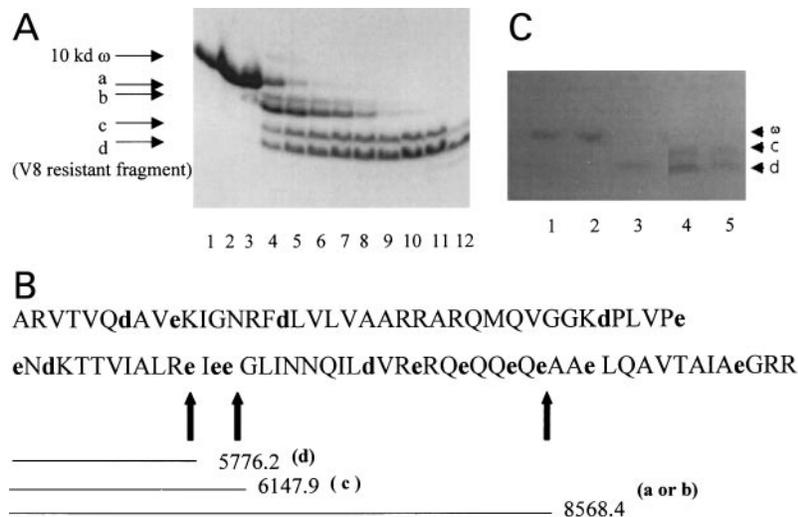
For endoproteinase Glu-C (V8) (Boehringer Mannheim) digestion reactions of  $\omega$ , V8 was added in a ratio of 1 : 200 (w/w) in 125 mM phosphate buffer (pH 7.8) containing 1 mM EDTA. Reactions were allowed to proceed at 25 °C for various time intervals and stopped by the addition of Laemmli loading buffer and immediate boiling. Products were analysed on 10% Tris/tricine gels [17] and stained with Coomassie blue. To determine the cleavage of  $\omega$  assembled in the polymerase, the proteins were transferred to Hybond-P (Amersham) and probed with antibody against  $\omega$ .

### Determination of V8 cleavage sites

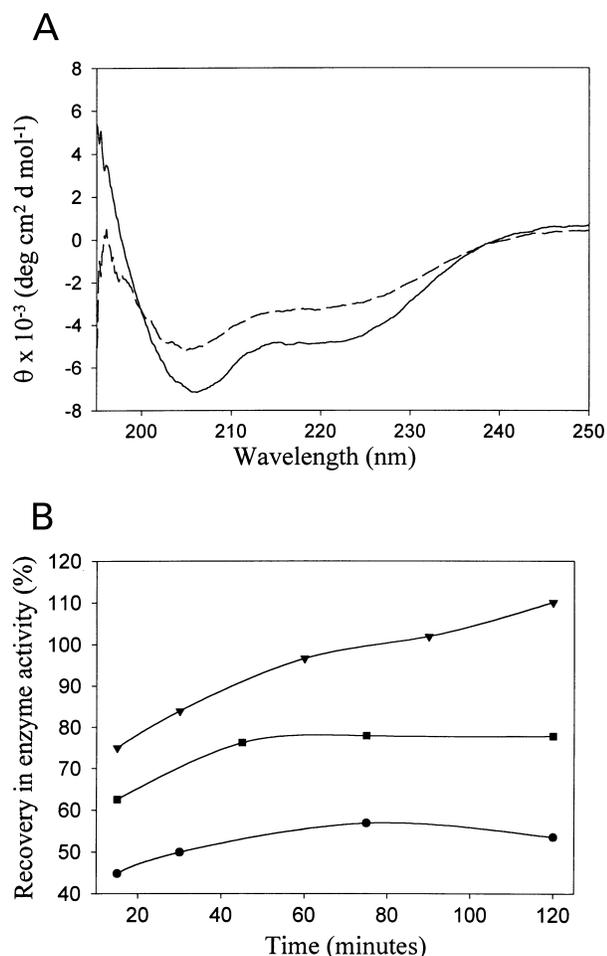
The cleavage products of  $\omega$  digested with V8 were transferred to a poly(vinylidene difluoride) membrane and the N-terminal amino-acid sequence of each product was determined. The molecular masses of purified cleavage products were determined on a Hewlett–Packard 1100 MSD electro-spray mass spectrometer.

### Analysis of secondary-structure content

Far-UV CD spectra of  $\omega$  and its 5.7-kDa fragment were recorded on a Jasco J-715 CD spectropolarimeter. Mean residue ellipticity  $[\theta] = (1000 \times \theta \times m)/Lc$ , where  $\theta$  is



**Fig. 1. Domain mapping of  $\omega$  with V8 protease.** (A) V8 digestion pattern of  $\omega$ : 40  $\mu$ g  $\omega$  was incubated with V8 in cleavage buffer (125 mM phosphate buffer, pH 7.8, containing 1 mM EDTA) at an  $\omega$  to V8 ratio (w/w) of 200 : 1 at 25 °C. At the time intervals indicated, an aliquot was removed and the reaction was stopped by boiling after addition of Laemmli loading dye; this was followed by electrophoresis on a 10% Tricine gel. Lane 1, undigested  $\omega$ ; lane 2,  $\omega$  without V8 incubated for 4 h; lanes 3–12, time intervals of 0, 5, 10, 15, 20, 30, 45, 60, 120, 240 and 270 min. (B) Schematic illustration of the  $\omega$  primary sequence and sites of V8 cleavage. The  $\omega$  fragments obtained were blotted on to poly(vinylidene difluoride) membranes and then stained with Coomassie Brilliant Blue R250. Stained protein bands on the poly(vinylidene difluoride) membranes were cut out and subjected to N-terminal amino-acid sequencing. Electrospray mass spectrometry (ES-MS) of each fragment was performed to determine their C-termini. Amino acids denoted in lower case represent the possible sites of V8 action. The arrows represent the sites of V8 action as determined by N-terminal sequencing and ES-MS. (C) V8 digestion of unassembled  $\omega$  and  $\omega$  assembled into RNA polymerase: 300  $\mu$ g wild-type RNA polymerase containing  $\omega$  and isolated 40  $\mu$ g  $\omega$  were treated with V8 at a protein to V8 ratio (w/w) of 200 : 1 for 2 h. The cleavage products were separated by electrophoresis on a 10% Tricine gel, blotted on to Hybond-P and probed with antibody against  $\omega$ . Lane 1, free  $\omega$ ; lane 2,  $\omega$  in wild-type polymerase; lane 3,  $\omega$ -NTD; lane 4,  $\omega$  digested with V8 (2 h); lane 5, RNA polymerase digested with V8 (2 h).



**Fig. 2. Secondary structure and function of  $\omega$ -NTD.** (A) Far-UV spectra of full-length  $\omega$  (solid line) and  $\omega$ -NTD (dashed line). (B) Reconstitution of RNA polymerase in the presence of  $\omega$  and  $\omega$ -NTD: 1 mg  $\omega$ -less RNA polymerase purified from the *rpoZ* null mutant was denatured in denaturation buffer containing 6 M urea. The denatured enzyme was refolded by removal of urea in the absence (●) or presence of a 10-fold molar excess of  $\omega$  (▼) or  $\omega$ -NTD (■). Aliquots were taken out at different time intervals and used for general transcription assay. The recovery of enzyme activity (%) was calculated by taking the activity of the native  $\omega$ -less enzyme as the reference.

the measured ellipticity in degrees,  $m$  is the mean residue weight in  $\text{g}\cdot\text{dmol}^{-1}$ ,  $c$  is the concentration in  $\text{g}\cdot\text{L}^{-1}$ , and  $L$  is the path length in cm.

### Assembly of $\omega$ into RNA polymerase

$\omega$ -less RNA polymerase purified from *rpoZ* null mutants was reconstituted with  $\omega$  and its 5.7-kDa fragment as described by Mukherjee & Chatterji [4]. Aliquots removed at different time points were used for multiple-round general transcription assay using calf thymus DNA as the template [18].

Reconstitution from individual subunits was performed by the method of Fujita & Ishihama [19], and the assembly intermediates were separated on a DEAE-cellulose column (Protein Pak G-DEAE from Waters) on the ÄKTA (Pharmacia) FPLC system. Individual peaks were analysed by SDS/PAGE (8% and 13% gels), followed

by Coomassie blue staining to identify  $\alpha$  dimer,  $\alpha_2\beta$  subassembly, core enzyme and the  $\omega$  subunit.

### Native gel electrophoresis

Equal amounts of  $\beta'$  in denaturing buffer was renatured by dialysis against reconstitution buffer in the presence of various amounts of  $\omega$ . After prolonged dialysis, the samples were centrifuged, and equimolar amounts were added to  $\alpha_2\beta$  subassemblies. The samples were incubated on ice for 30 min and then at 30 °C for an additional 30 min; they were then separated on a 4% native Tris/borate/polyacrylamide gel and silver stained.

### Ni/nitrilotriacetic acid/agarose column to assay binding of $\beta'$ to $\omega$

Binding of  $\omega$  and  $\omega$ -NTD to  $\beta'$  was assayed on a Ni/nitrilotriacetic acid column as previously described [16] and analysed by SDS/PAGE on 5–20% gradient gels (Bio-Rad).

### Aggregation assays with $\beta'$

Denatured  $\beta'$  in 6 M urea was diluted with 50 mM Tris/HCl, pH 8.0, to a concentration of 5  $\mu\text{M}$  and the aggregation of  $\beta'$  was monitored by measuring the light scattered at 360 nm over a time period of 5 min. The ability of  $\omega$  and  $\omega$ -NTD to prevent  $\beta'$  aggregation was followed by diluting  $\beta'$  in 50 mM Tris/HCl, pH 8.0, containing various amounts of  $\omega$  or  $\omega$ -NTD. Subsequently, the aggregation of  $\beta'$  was measured over 5 min as a function of increase in turbidity at 360 nm.  $\beta'$  aggregation was also monitored in the presence of equivalent amounts of BSA,  $\alpha$ ,  $\beta$  and GroEL as controls. Denatured  $\beta'$  after renaturation in the presence of different amounts of  $\omega$  was centrifuged at 30 000 g for 30 min, and the supernatant was loaded on an SDS/8% polyacrylamide gel to determine the amount of  $\beta'$  recovered in the soluble phase.

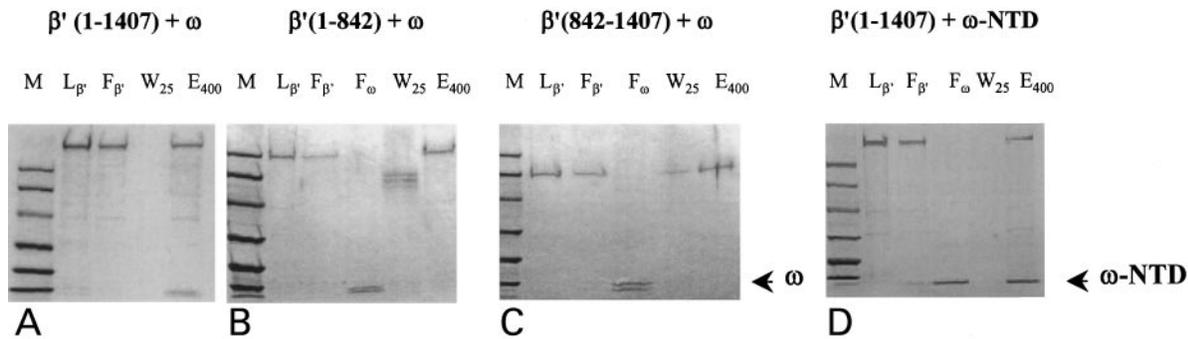
## RESULTS

### $\omega$ consists of a V8-resistant NTD followed by an unstructured chain

Limited proteolysis is a classical method for defining domain organization in a protein [20–24], as proteases require unstructured substrates and consequently tend not to cut within the domains. Endoproteinase GluC (V8), which cleaves after glutamate and aspartate residues, was used in the limited digestion of  $\omega$  to gain an insight into the structure of this subunit. Figure 1A shows the digestion pattern of  $\omega$  when incubated with V8 under nondenaturing

**Table 1. Identification of  $\omega$  fragments upon V8 digestion.**

Band	N-Terminal sequence	Observed mass (Da)	$\omega$ residues	Calculated mass (Da)
$\omega$	ARVTVQD	10105	1–90	10105
a	ARVTVQD	8568.36	1–75	8567.65
b	ARVTVQD			
c	ARVTVQD	6147.96	1–55	6146.04
d	ARVTVQD	5776.24	1–52	5774.65



**Fig. 3.** Binding of  $\omega$  and  $\omega$ -NTD to  $\beta'$  and  $\beta'$  fragments assayed on a Ni/nitrilotriacetic acid column.  $\beta'$ -His<sub>6</sub> or His<sub>6</sub>-tagged  $\beta'$  fragments in dissociation buffer were mixed with Ni/nitrilotriacetic acid/agarose equilibrated with the same dissociation buffer. After a 1-h incubation at 4 °C, the beads were washed with reconstitution buffer and then with buffer D [50 mM Tris/HCl, pH 7.9, at 4 °C, 0.1 mM EDTA, 5% (v/v) glycerol] plus 25 mM imidazole. The proteins were eluted with buffer D plus 400 mM imidazole. Each fraction was analysed by SDS/PAGE on 5–20% gradient gels (Bio-Rad Ready Gels J). Lanes M, low-molecular-mass marker (Pharmacia); lanes L, applied  $\beta'$  samples; lanes F, unbound fractions; lanes W, wash fraction with buffer + 25 mM imidazole; lanes E, elution fraction with buffer + 400 mM imidazole.

conditions over 4 h. Control experiments showed that V8 was stable over the time course of the experiments and did not undergo self-digestion (data not shown). Self-digestion of V8 was also prevented by carrying out the reaction at 25 °C.  $\omega$  is cleaved into four discrete fragments (a–d), of which only one remained stable on prolonged digestion (Fig. 1A). A combination of N-terminal sequencing and electron-spray MS analysis, along with a consideration of the cleavage specificity of V8 (C-terminus of Glu or Asp residues), was used to identify the products of  $\omega$  digestion precisely. The results are summarized in Fig. 1B and Table 1.

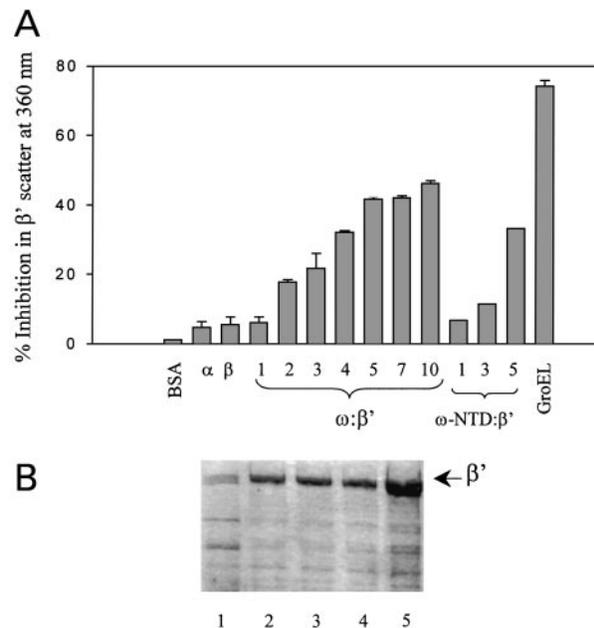
All four bands (a–d) were found to have the same N-terminal sequence corresponding to the N-terminal sequence of the full-length protein, implying that  $\omega$  is cleaved by V8 sequentially from the C-terminus. The cleavage products were purified to homogeneity using RP-HPLC. We were unable to resolve bands a and b by the above process. MS analysis of the purified peptides revealed that one of the initial points of cleavage was after Glu75, leading to the appearance of the 8576-Da fragment (either a or b). This was then cleaved further to give rise to a 6147-Da fragment (c) corresponding to cleavage after Glu55. A 5776-Da V8-resistant fragment (d) was finally formed by cleavage after Glu52. We therefore infer that  $\omega$  consists of an NTD comprised of 52 amino acids; the region beyond this is highly unstructured making it highly accessible to the protease.

We have also studied the accessibility of  $\omega$  to the V8 protease when it is assembled in the RNA polymerase holoenzyme. Figure 1C shows the pattern of V8 digestion of unassembled  $\omega$  and when it is associated with the other subunits in the holoenzyme. In both the cases, a 5.7-kDa fragment was formed which was not cleaved further by V8. This indicates that the structure of  $\omega$  does not undergo any significant change on its assembly into the enzyme.

### The NTD of $\omega$ has a defined secondary structure

Next, the secondary-structure content of  $\omega$ -NTD was analysed using far-UV CD spectroscopy. The far-UV spectrum shows that the  $\omega$ -NTD has a well-defined secondary structure (Fig. 2A), which is very similar to

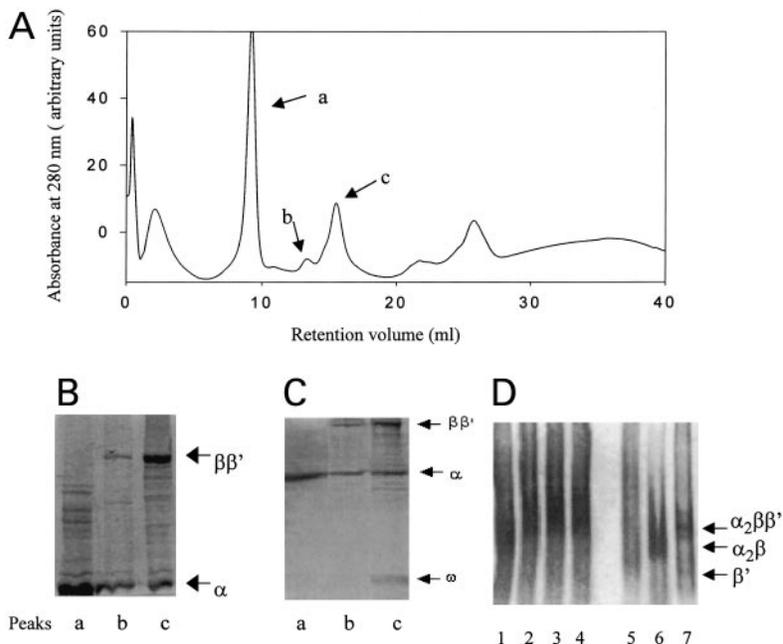
that of the full-length  $\omega$ , indicating that most of the secondary structure of  $\omega$  is contained within this domain. This is consistent with the V8 digestion pattern, which shows that the C-terminal proximal region after the NTD is unstructured.



**Fig. 4.** Inhibition of  $\beta'$  aggregation by  $\omega$  and  $\omega$ -NTD. (A)  $\beta'$  denatured in 6 M urea was renatured by quick dilution of urea by the addition of renaturation buffer (50 mM Tris/HCl, pH 8.0) to a concentration of 5  $\mu$ M. Aggregation of  $\beta'$  was monitored by measuring the turbidity at 360 nm over 5 min. Refolding of  $\beta'$  was studied in the presence of different concentrations of  $\omega$  and  $\omega$ -NTD. (B) Denatured  $\beta'$  (5  $\mu$ M) was refolded in the presence of different amounts of  $\omega$ . The samples were centrifuged at 30 000 g, and the supernatant was subjected to SDS/PAGE (8% gel) to detect the  $\beta'$  recovered in the soluble phase. Lane 1, amount of  $\beta'$  recovered when refolded in the absence of  $\omega$ ; lanes 2–4, amount of  $\beta'$  recovered when refolded in the presence of twofold, threefold and fourfold molar excess of  $\omega$ ; lane 5, initial amount of denatured  $\beta'$  used for refolding.

**Fig. 5. Reconstitution of *E. coli* RNA polymerase from its constituent subunits.**

(A) RNA polymerase subunits were mixed and denatured in denaturation buffer containing 6 M urea and refolded by dialysing out the urea (see Materials and methods). The subassemblies were separated on a DEAE-cellulose column using a 0.2–0.7 M NaCl gradient. (B) Individual peaks a ( $\alpha_2$ ), b ( $\alpha_2\beta$ ) and c ( $\alpha_2\beta\beta'$ ) were subjected to SDS/PAGE (8% gel) followed by Coomassie blue staining. (C) Individual peaks a ( $\alpha_2$ ), b ( $\alpha_2\beta$ ) and c ( $\alpha_2\beta\beta'$ ) were subjected to SDS/PAGE (13% gel) followed by Coomassie blue staining. (D) 5  $\mu$ M  $\beta'$  was renatured in the presence of various amounts of  $\omega$ . The samples were centrifuged and added to equimolar amounts of  $\alpha_2\beta$ . The conversion of  $\alpha_2\beta$  to  $\alpha_2\beta\beta'$  was monitored on a 4% native polyacrylamide gel. Lane 1,  $\beta'$  renatured in the absence of  $\omega$ ; lanes 2–4,  $\beta'$  renatured in the presence of twofold, threefold, or fourfold molar excess of  $\omega$ ; lane 5, free  $\beta'$ ; lane 6,  $\alpha_2\beta$ ; lane 7,  $\alpha_2\beta\beta'$  core.



#### $\omega$ -NTD retains the function of the full-length protein

On reconstitution of RNA polymerase, the maximum recovery of the activity of denatured RNA polymerase occurs in the presence of  $\omega$  [4]. To ascertain the behaviour of  $\omega$ -NTD, the renaturation profile of denatured  $\omega$ -less enzyme in the presence of  $\omega$ -NTD was measured. Figure 2B shows the time-dependent recovery of RNA polymerase activity after renaturation in the absence or presence of either intact  $\omega$  or  $\omega$ -NTD. The results indicate that  $\omega$ -NTD is capable of restoring the activity of denatured  $\omega$ -less enzyme, albeit to a lesser extent than intact  $\omega$ . It is possible that the C-terminal region may be involved in the binding of  $\omega$  and thereby affect the activity of the NTD.

#### Binding of $\omega$ and $\omega$ -NTD to $\beta'$ -subunit

It has previously been shown that  $\omega$  cross-links with  $\beta'$  in the RNA polymerase holoenzyme [9]. Here we attempted to identify the domain of  $\beta'$  that interacts with the  $\omega$  subunit. For this purpose, we used a series of His<sub>6</sub>-tagged  $\beta'$  fragments cloned in order to decipher the subunit-subunit contact surfaces on the  $\beta'$  subunit [16]. On Ni/nitrilotriacetic acid affinity chromatography,  $\omega$  was found to be coeluted with intact  $\beta'$ -His<sub>6</sub> at 400 mM imidazole, indicating high affinity between the two proteins (Fig. 3A). We then examined the  $\omega$ -binding activity of two  $\beta'$  fragments,  $\beta'$ -NTD(1–842)-His<sub>6</sub> and  $\beta'$ -CTD(842–1407)-His<sub>6</sub>. Interestingly, when  $\beta'$ -NTD-His<sub>6</sub> or  $\beta'$ -CTD-His<sub>6</sub> were immobilized and subsequently incubated with  $\omega$ , all the  $\omega$  remained unbound and was eluted with the low imidazole wash (Fig. 3B,C). Binding of  $\omega$  was also not detected on immobilizing  $\beta'$ -NTD and  $\beta'$ -CTD together on the Ni/nitrilotriacetic acid column (data not shown). Thus we inferred that the entire  $\beta'$  structure is necessary for tight binding of the  $\omega$  subunit.  $\omega$ -NTD

was found to have similar  $\beta'$ -binding properties to those of intact  $\omega$  (Fig. 3D). It is possible that a well-defined middle segment of  $\beta'$ , disrupted in either half of the protein, is responsible for interaction with  $\omega$ . However, this seems unlikely from the crystal structure [12], which shows  $\omega$ -interacting regions to be present entirely in the N-terminal (residues 755–762) and C-terminal (residues 1216–1220 and 1476–1486) portions of  $\beta'$ .

#### Renaturation of $\beta'$ subunit with $\omega$ and $\omega$ -NTD

Denatured  $\beta'$  tends to aggregate during its refolding at micromolar concentrations. This phenomenon commonly occurs during protein refolding as the result of multi-molecular interchain interactions, which increase with protein concentration, leading to the formation of trapped intermediates and consequent aggregation [25]. Chaperones have evolved to prevent the formation of such aggregates [26,27]. As  $\beta'$  is the only RNA polymerase subunit that does not associate with GroEL *in vivo* [10] and is the only subunit to cross-link with  $\omega$  [9], we studied the ability of  $\omega$  to prevent such aggregation of  $\beta'$ . Light scattering at 360 nm by the aggregated fraction of  $\beta'$  was taken as a measure of the degree of aggregation inhibition. Figure 4A shows the percentage inhibition of  $\beta'$  scatter with increasing  $\omega$  concentration.  $\omega$ -NTD also showed a similar trend in preventing the aggregation of the  $\beta'$  subunit. However, addition of BSA, RNA polymerase  $\alpha$  and  $\beta$  subunits as controls did not inhibit  $\beta'$  aggregation. We could not achieve an inhibition of more than 50% by the addition of  $\omega$ . However, purified GroEL could almost completely inhibit  $\beta'$  aggregation ( $\approx 80\%$ ). (We will discuss this apparent discrepancy in the *in vitro* and *in vivo* results in a later section.) We also studied the amount of  $\beta'$  recovered in the soluble phase as a function of  $\omega$  concentration. Denatured  $\beta'$  was renatured in the

presence of different concentrations of  $\omega$ . The samples were centrifuged to remove aggregated  $\beta'$  and the amount of  $\beta'$  recovered in the supernatant was monitored by SDS/PAGE (8% gel). Figure 4B shows an increasing amount of  $\beta'$  in the soluble phase with an increase in the  $\omega$  concentration.

### Reconstitution of RNA polymerase

RNA polymerase can be reconstituted *in vitro* from the individual subunits and the intermediate subassemblies are separated by passing through a DEAE-cellulose column as standardized previously [19]. Figure 5A shows the elution profiles of the  $\alpha_2$ ,  $\alpha_2\beta$  and  $\alpha_2\beta\beta'$  fractions from a DEAE-cellulose column when excess  $\alpha$  subunit was used. When reconstitution from individual subunits was carried out in the presence of  $\omega$ ,  $\omega$  was found to be incorporated only during the association of  $\alpha_2\beta$  with  $\beta'$  (Fig. 5C). Neither the dimeric  $\alpha_2$  nor the  $\alpha_2\beta$  subassembly contained detectable amounts of the  $\omega$  subunit. Quantitation of the gel in Fig. 5C using Quantity One (Bio-Rad) indicated the presence of a 20-fold excess of  $\omega$  in  $\alpha_2\beta\beta'$  (lane c) compared with  $\alpha_2\beta$  (lane b) when the intensities were normalized with respect to  $\alpha$  in lane b. The presence of  $\omega$  in only the  $\alpha_2\beta\beta'$  subassembly indicates that  $\omega$  is incorporated in the final stage of assembly, concomitant with the incorporation of  $\beta'$  into the  $\alpha_2\beta$  subassembly.

We also studied the conversion of  $\alpha_2\beta$  into the  $\alpha_2\beta\beta'$  core by native PAGE. We previously reported that different forms of core RNA polymerase, with or without  $\omega$ , can be separated by native PAGE [5]. Denatured  $\beta'$  was renatured in the presence of various amounts of  $\omega$ . The soluble  $\beta'$  fraction was then added to a fixed amount of  $\alpha_2\beta$ . Figure 5D shows that formation of the  $\alpha_2\beta\beta'$  core is promoted when the added  $\beta'$  is renatured in the presence of  $\omega$ .

## DISCUSSION

The  $\omega$  subunit was previously thought to be involved in stringent regulation in *E. coli* because of the presence of *rpoZ*, encoding  $\omega$ , in the same operon as *spoT*, which codes for the product responsible for the synthesis and degradation of ppGpp in *E. coli* [28]. However, it was later observed that stringent control of stable RNA synthesis was unaffected by an *rpoZ* null allele [29], raising further questions about the function of  $\omega$  *in vivo*. The only phenotype of slow growth shown by the *rpoZ*<sup>-</sup> strain of *E. coli* was thought to be due to the strong polar effect on *spoT* located downstream of *rpoZ* [3]. However,  $\omega$ -less RNA polymerase shows a strong association with GroEL [5]. This, along with our finding that  $\omega$  helps in the renaturation of denatured RNA polymerase [4], prompted us to assign a chaperone-like function for  $\omega$ . However, the step at which  $\omega$  is involved in RNA polymerase assembly and its specific role was largely unknown.

Through a series of elegant experiments, Houry *et al.* [10] identified a group of GroEL substrates in *E. coli* *in vivo*. They proposed that GroEL strongly interacts with a well-defined set of  $\approx 300$  newly translated polypeptides, including essential components of the transcription machinery. Interestingly, all the components of core RNA polymerase ( $\alpha$ ,  $\beta$ ,  $\omega$ ) except  $\beta'$  are substrates of GroEL. This, along with our observation that it is very

difficult to resolubilize  $\beta'$  during its purification from inclusion bodies, prompted us to undertake this study. The experiments presented here demonstrate the ability of  $\omega$  to prevent the aggregation of  $\beta'$  and yield a stoichiometry of  $\omega$  to  $\beta'$  much higher than 1 : 1 as expected from the X-ray structure of *T. aquaticus* RNA polymerase [11]. We could not immediately ascribe a reason for this apparent discrepancy. It appears that a large amount of  $\omega$  is required to keep  $\beta'$  in a soluble form *in vitro*. Whether the same occurs *in vivo* is a subject of speculation. Were the same requirement necessary *in vivo*, we would expect release of the excess  $\omega$  on association of  $\beta'$  with the  $\alpha_2\beta$  subassembly. The presence of an inactive fraction of  $\omega$  also cannot be completely ruled out. It is, however, noteworthy that no subunit other than  $\omega$  is capable of preventing  $\beta'$  aggregation *in vitro*. The aggregation of  $\beta'$  was also found to be prevented by GroEL despite the fact that  $\beta'$  is not an *in vivo* substrate of GroEL. This is not surprising as GroEL is known to interact with almost any nonnative model protein [30], although *in vivo* it is involved in the folding of only  $\approx 10\%$  of the newly translated polypeptides [31].

When core RNA polymerase is denatured and subsequently reconstituted *in vitro*, the enzyme assembles in a stepwise manner to form the  $\alpha_2$ ,  $\alpha_2\beta$  and  $\alpha_2\beta\beta'$  subassemblies [8]. Figure 5 shows that  $\omega$  is included along with  $\beta'$  in the  $\alpha_2\beta$  subassembly. Moreover, the entire  $\beta'$  subunit is required for binding to  $\omega$ , as neither the NTD nor the CTD alone can bind  $\omega$  (Fig. 3). These results, together with the observation that  $\omega$  can prevent  $\beta'$  aggregation during its renaturation *in vitro* (Fig. 4) suggest that  $\omega$  binds the NTD and CTD of  $\beta'$  simultaneously, maintains it in an aggregation-resistant conformation, and recruits it to the  $\alpha_2\beta$  subassembly. Our results are consistent with the recent structural analysis of the  $\beta'$ - $\omega$  interface [12], which predicts that the conserved regions in  $\omega$  make simultaneous contacts with the NTD and CTD of  $\beta'$  and promotes assembly of RNA polymerase by binding to the largest subunit. Moreover,  $\alpha$ -helix 2 in CR1 of  $\omega$  contributes to most of the interactions between  $\omega$  and  $\beta'$ , making contacts with both the N-terminal region and the C-terminal tail of  $\beta'$ . The C-terminal region of  $\omega$  also makes additional interactions with the C-terminal tail of  $\beta'$ , although our results on  $\omega$ -NTD presented here suggest that the interactions made by helix-2 (CR1; residues 15–34 in *E. coli*) are sufficient for the function of  $\omega$ .

Although residues 35, 40, 41, 43, 54, 64, 67, 78 and 87 lie within the unstructured regions of  $\omega$  assembled in the polymerase (see [12]), these residues are not acted on by V8 in the unassembled protein. We had expected a change in  $\omega$  structure upon its assembly into the polymerase. However, unassembled free  $\omega$  and  $\omega$  assembled in the polymerase show similar V8 digestion patterns, indicating no major structural change in  $\omega$  after its association with RNA polymerase. In any case, a difference in the structure of  $\omega$  in *T. aquaticus* and *E. coli* cannot be ruled out, and a detailed structural analysis of *E. coli*  $\omega$  will answer this point.

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