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An Improved Procedure for the Purification of the *Escherichia coli* RNA Polymerase Omega Subunit

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Abstract

We report an improved procedure for purification of the omega subunit of *Escherichia coli* RNA polymerase. In contrast to the original procedure, the revised procedure (i) allows purification of omega entirely from the soluble fraction, obviating the need for denaturation/renaturation, (ii) results in >99% pure omega in only 2 chromatographic steps, and (iii) improves the yield of purified omega by at least 5-fold. Reconstitution of *E. coli* RNAP from omega purified by this procedure, as well as purified sigma and core RNAP lacking omega, produces active holoenzyme *in vitro*, and co-overexpression of omega from a plasmid containing *rpoZ* and an additional plasmid encoding the other RNAP core subunits results in production of active core enzyme *in vivo*.

Keywords

RNA Polymerase; Omega; *rpoZ*; Red-Agarose

Introduction

The ~400 kDa *Escherichia coli* RNA polymerase (RNAP) core enzyme is composed of two α subunits and one subunit each of β , β' , and ω [1]. The α dimer is the scaffold for assembly of the other subunits, but the bulk of the enzyme, including the enzyme's active site, is composed of β and β' . The core enzyme assembles with one of the seven *E. coli* sigma factors to form RNAP holoenzyme which is capable of binding specifically to promoter DNA and initiating transcription. The smallest of the subunits, ω , at only 10,105 Da (90 amino acids, [2]) has drawn interest of late because of recent discoveries of its roles in RNAP assembly and in enzyme regulation.

ω is conserved not only in bacteria, but also in archaea (RpoK) and eukaryotes (Rpb6) [3]. ω appears to facilitate RNAP assembly, specifically by aiding β' subunit binding to the $\alpha_2\beta$ subassembly [3–5]. In support of a role for ω in RNAP formation, RNAP assembled in the absence of ω displays greater association with molecular chaperones like GroEL *in vivo* [6].

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Nevertheless, holoenzyme can assemble *in vitro* in the absence of ω , the enzyme is competent for transcription *in vitro* and *in vivo*, and *E. coli* cells lacking *rpoZ* (the gene coding for ω) are viable [7,8].

An early study [9] suggested that ω might play a role in the response of RNAP to the effectors of the stringent response, guanosine tetraphosphate and pentaphosphate (here referred to as ppGpp). However, subsequent work showed that cells lacking the *rpoZ* gene still exhibited stringent control *in vivo*, indicating that they still responded to ppGpp [8]. More recently, we demonstrated that ω is required for *E. coli* RNAP to respond to ppGpp *in vitro* [10], but *in vivo* the presence of DksA, another RNAP-binding protein factor, can compensate for the absence of ω . Although this potentially explained the seeming contradiction that ppGpp was needed for responses of *E. coli* RNAP to ppGpp *in vitro* but not *in vivo*, not all bacterial species appear to contain DksA. Thus, some other bacteria may require ω for ppGpp-dependent regulation *in vivo*. In support of this model, *Streptomyces kasugaensis* strains deleted for *rpoZ* display a defect in antibiotic production, which requires ppGpp [11,12].

Given the requirement for inclusion of ω in RNAP preparations and the recent interest in examining the role of ω in RNAP assembly and function, we sought to improve ω production and purification. Because of its small size, as well as the interactions of its protein termini with other RNAP subunits and therefore the potential that even small extension(s) might interfere with ω function, we chose not to employ epitope tagging and affinity purification for ω purification.

Here, we report a revised procedure for purification of overexpressed, untagged *E. coli* ω . The revised procedure, which employs the plasmid pCDF- ω rather than the previously-used pE3C for overproduction [13], has several significant advantages compared to the original. (i) The purification is achieved in only two column steps compared to the original three-column procedure. (ii) The procedure eliminates a requirement for ω denaturation/renaturation. (iii) The procedure results in a significantly higher yield of ω . We also show that Red-Agarose is an acceptable substitute for the Red-Sepharose resin used in the original protocol. This is important as it appears that Red-Sepharose no longer is available commercially.

Materials and methods

Construction of pCDF- ω

pCDF- ω was obtained from R.H. Ebright [10]. It was constructed from pCDFDuet-1 (Novagen), a CloDF13 replicon that encodes streptomycin/spectinomycin resistance. The *rpoZ* gene was PCR amplified using primers that created an NdeI restriction site immediately upstream of the *rpoZ* start codon and an XhoI restriction site immediately downstream of the *rpoZ* stop codon. The PCR product was inserted into pCDFDuet-1 multiple cloning site 2, between the NdeI and XhoI restriction sites, resulting in transcription of *rpoZ* under the control of a T7 promoter and production of untagged ω protein.

Overexpression of ω from pCDF- ω and preparation of cell lysates

Overproduction of ω from pCDF- ω was performed in BL21(DE3), which lacks OmpT and produces T7 RNAP under the control of the *lac* promoter/operator. It was reported previously that OmpT causes proteolysis of ω [13]. BL21(DE3) cells carrying pCDF- ω (RLG7817) were plated on LB-spectinomycin (50 μ g/ml) agar, and cells directly from the plate were used to inoculate 1–2 liters of LB-spectinomycin to an OD₆₀₀ of 0.01. At an OD₆₀₀ of 0.4–0.6, T7 RNAP expression and ω production were induced with 1 mM IPTG, and after 2.5 hr, cells were harvested by centrifugation, wet weights were recorded (~2–2.5 g of cells per liter of culture), and pellets were stored at –80°C. Thawed cell pellets (2.4 g of wet weight cells) were

resuspended in 7.2 ml grinding buffer (50 mM Tris HCl pH 8.0, 2 mM EDTA, 50 mM NaCl, 1 mM PMSF), and cells were lysed by incubation for 15 min with lysozyme (0.5 mg/ml, room temperature) and for an additional 15 min with Triton X-100 (1%). This was followed by sonication (5 × 30 sec on ice, 1 min on ice between each sonication) and centrifugation (18,000 × g, 30 min), and the supernatant was recovered.

Visualization and purification of ω

ω does not absorb at 280 nm because it lacks tryptophan and tyrosine residues [2,13]. Therefore, ω was detected by PAGE on either 12% or 4–12% Nu PAGE Bis-Tris gels (Invitrogen). MES electrophoresis buffer was used for the 4–12% gels in order to improve resolution of ω and other lower molecular weight proteins. All protein samples were mixed with 5X LDS Sample Preparation Buffer (Invitrogen) and heated at 95°C for 1 min before gel loading. Protein concentrations were determined using the Bradford assay reagent (BioRad), using BSA as a standard.

Chromatography with Q-Sepharose was performed essentially as described [13]. The sonicate (7.2 ml; see above) was diluted with 50 mM Tris-HCl, pH 8.0 to ~10 mM NaCl, passed through a 0.45 μ m filter, and loaded on a 50 ml Q-Sepharose Fast Flow column (GE Healthcare) equilibrated with 50 mM Tris-HCl pH 8.0 (flow rate, 4 ml/min). The column was washed with buffer until absorbance at 280 nm stopped decreasing, and then 3–4 ml fractions were collected from elution with a linear gradient of 0–0.5 M NaCl in 50 mM Tris buffer. The column was then stripped with high salt (1 M NaCl) buffer to recycle it for further use. Fractions containing ω were pooled as indicated in the legend for Fig. 2 and loaded on a 10 ml Red-Sepharose (Pharmacia) or Red-Agarose (Sigma) column equilibrated with 50 mM Tris HCl pH 8.0 and 0.2 M NaCl (flow rate, 2 ml/min). After washing with 5 column volumes of 50 mM Tris HCl pH 8.0, 0.2 M NaCl, 2 ml fractions were collected from elution with 50 mM Tris HCl pH 8.0, 1.0 M NaCl. Fractions containing ω as judged by PAGE (see above) were pooled and dialyzed overnight against 2 liters of storage buffer (25 mM Tris HCl pH 8.0, 50 mM NaCl, 50% glycerol) with one change and stored at –80° C. Growth of cells at either 30° or 37° C produced roughly equivalent yields of overexpressed ω .

Results and Discussion

Overexpression of ω from pCDF- ω

Induction with IPTG for 2.5 hr resulted in overproduction of ω from pCDF- ω (Fig. 1A, lane 1 vs. lane 2). We estimate that ω accounted for ~15% of total cell protein, in contrast to overexpression from pE3C which was reported to produce ω as only 1–5% of total cell protein [13]. The BL21(DE3) (pCDF- ω) expression system did not require heat induction or a second source of T7 RNAP, in contrast to the previous expression system.

The majority of the ω produced in the original ω purification protocol went into the insoluble fraction, which necessitated solubilization with Sarkosyl and renaturation by dialysis. In contrast, the vast majority of ω produced in the BL21(DE3) (pCDF- ω) expression system remained in the soluble fraction (Fig. 1B, lane 2 vs. lane 3). Similar results were obtained in three additional independent experiments (data not shown).

Step 1: Purification by Q-Sepharose

The soluble fraction was applied to a Q-Sepharose ion-exchange column as an initial purification step. From the absorbance profile at 280 nm, most protein eluted late in the salt gradient (Fig. 2A), but from the gel analysis (and as reported previously by Gentry and Burgess [13]), ω eluted early (Fig. 2B, arrow). Thus, pooling of fractions 13–16 eliminated a large

proportion of the contaminants, including virtually all proteins over 40 kDa in size, while still resulting in recovery of large amounts of the overexpressed ω .

Step 2: Purification by Red-Sepharose or Red-Agarose

In accord with the observations of Gentry and Burgess [13], Procion Red-Sepharose preferentially retained ω , whereas virtually all other proteins flowed through the column, and addition of 1 M NaCl resulted in efficient ω elution (Fig. 3A). Fractions 5–7 were pooled and dialyzed into storage buffer (see above) and stored at -80°C . The resulting ω was >99% pure and ready for assembly with the other RNAP subunits, in contrast to the previous protocol which required an additional ion-exchange chromatography step to achieve an equivalent level of purity.

We were unable to identify a commercial source of Red-Sepharose. To determine whether Red-Agarose (which is also available as a Procion red dye-linked resin) would substitute for Red-Sepharose, the ω -containing Q-Sepharose fractions were applied to a Red-Agarose column, using the same salt conditions for binding and elution as for the Red-Sepharose column shown in Fig. 3A. Fig. 3B shows that the Red-Agarose column produced virtually identical results as the Red-Sepharose column.

We emphasize that we sacrificed some of the ω -containing fractions at the Q-Sepharose step for the sake of increased purity. Nevertheless, our yields of purified ω were still significantly higher than those reported previously for equivalent amounts of starting material [13]. We recovered ~1 mg of > 99% pure ω / gram wet cell weight with Red-Agarose (approximately as much as obtained using Red-Sepharose; data not shown). In contrast, Gentry and Burgess [13] recovered ~0.2 mg pure ω /gram wet cell weight, of which ~75% came from the insoluble fraction and had to be refolded.

Purification of ω from later Q-Sepharose fractions

We also pooled fractions 17–25 from the Q-Sepharose column (Fig. 2B) and purified these over Red-Agarose. Most of the non- ω contaminants did not bind to the Red-Agarose resin and were found in the flowthrough fractions (data not shown). However, several higher molecular weight proteins also contaminated the ω -containing fractions, eluting from Red-Agarose with ω at high salt (Fig. 4A, 4B). We estimate that the yield of ω might be as much as doubled by utilizing these ω -containing Q-Sepharose fractions, but >99% purity would require a third purification step (i.e., after the Q-Sepharose and Red-Agarose steps), such as a Mono-Q chromatography step like that included by Gentry and Burgess [13].

Activity and yield of purified ω

Our procedure for purification of *E. coli* ω results in high yield in two column steps using a standard T7 RNAP-based expression system. One liter of bacterial culture yielded ~290 mg of total protein, from which we recovered ~2.1 mg of ω of >99% purity. We also recovered ~2.2 mg of ω at slightly lower (~95%) purity from the same lysate. Thus, the total yield of ω was ~10%, assuming that the overexpressed ω represented ~15% of total protein in the starting material (see Fig. 1). The procedure does not require heat induction (i.e., expression can be performed at 30° or 37°C), and it produces ω in the soluble cell fraction, thereby eliminating the need for solubilization with Sarkosyl and extensive dialysis. We estimate that the amount of ω obtained from one liter of overexpressing cells would be sufficient to supplement >15,000 individual 10 μl reactions using our *in vitro* transcription conditions and RNAP lacking ω .

It was shown previously that ω purified by the original procedure was active in binding to RNAP [13]. We showed previously that the ω obtained using the revised purification procedure not only bound to RNAP but also restored the sensitivity of RNAP lacking ω to the small

molecule regulator, ppGpp [10,14]. Quantitative comparison of the activity of ω made by the original procedure with that made by the revised procedure would require a supply of Red-Sepharose (which is no longer available) and measurement of effects of ppGpp on transcription at different levels of saturation of RNAP with ω . However, because the revised purification protocol produced ω that conferred the same degree of inhibition by ppGpp on RNAP holoenzyme reconstituted *in vitro* as did ω assembled with native RNAP holoenzyme *in vivo* [10], we conclude that the revised protocol provides ω that operationally is likely to be at least as active as that made by the original procedure. (Fig. 5)

Use of pCDF- ω for producing ω -saturated RNAP *in vivo*

We have shown previously that ω overexpressed from pCDF- ω assembles to form active core RNAP when co-overexpressed with the other core RNAP subunits ($\alpha_2\beta\beta'$) *in vivo* [10,14]. In fact, the core enzyme produced in this way appears to be slightly more saturated with ω than core enzyme from standard RNAP purifications (data not shown). However, we note that the yield of $\alpha_2\beta\beta'$ is lower in the presence of pCDF- ω than in the absence of pCDF- ω (data not shown), most likely because there is competition for limiting T7 RNAP among the T7 promoters on pCDF- ω and on the plasmid encoding $\alpha_2\beta\beta'$ (pIA299) [15].

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Figure 1. Overproduction of ω from BL21(DE3) (pCDF- ω)

(A) Protein lysate from cells without induction (lane 1) or after addition of 1 mM IPTG to cells for 2.5 hr (lane 2). Cell samples were pelleted, resuspended in loading dye, boiled, separated on a 4–12% Novex Bis-Tris gel (MES running buffer; Invitrogen), and proteins were stained with Coomassie Blue. (B) Protein lysate after induction with IPTG as in (A) without fractionation by centrifugation (lane 1). Soluble fraction (supernatant; lane 2). Insoluble fraction (pellet; lane 3).

Figure 2. Purification by Q-Sepharose

Cell lysates were passed over a Q-Sepharose Fast Flow column and eluted with buffer containing a linear salt gradient (0–0.5 M NaCl) as described in Methods. (A) Conductivity and absorbance at 280 nm are indicated. The absorbance derives from the impurities, since ω does not absorb at 280 nm. Numbers below the X-axis refer to ml of column effluent. Black and gray bars below the X-axis indicate fractions 13–16 and 17–25, respectively, pooled for further processing as described in the text and illustrated in Fig. 5. (B) Fractions shown in (A) were analyzed by PAGE on a 4–12% Bis-Tris gel and stained with Coomassie Blue. SeeBlue Plus2 protein marker ladder (Invitrogen) is on left (M).

Figure 3. Purification of ω with Red-Sepharose or Red-Agarose

Pooled fractions from the Q-Sepharose column (Fig. 2) were applied to either Red-Sepharose (A) or Red-Agarose (B) equilibrated in 50 mM Tris pH 8.0, 0.2 M NaCl. ω was eluted with 1 M NaCl (2 ml fractions) and separated by PAGE on a 12% Bis-Tris gel (A) or a 4–12% Bis-Tris gel (B) and stained with Coomassie Blue. In each case, fractions 5–7 were pooled for use.

Figure 4. Purification of ω from other Q-Sepharose fractions on Red-Agarose

Pooled fractions 17–25 (Fig. 2) were applied to Red-Agarose as in Fig. 3 and eluted with 1.0 M NaCl. (A) Absorbance and conductivity traces from Red-Agarose column are shown. The absorbance derives from the impurities, since ω does not absorb at 280 nm. The vertical arrow in Fig. 4B refers to the position of fraction 5, and numbers below the X-axis represent ml of column eluant. The gray bar under the X-axis corresponds to fractions 2–10 shown in Fig. 4B. The salt concentrations at the beginning (0.2 M NaCl) and end (1 M NaCl) of the step elution are indicated. (B) Fractions in (A) were analyzed by PAGE on a 4–12% Bis-Tris gel and stained with Coomassie Blue.

Figure 5. Flow-chart of ω purification procedure

Only one additional chromatographic step (Red-Agarose or Red-Sepharose) was required for >99% purity from Q-Sepharose fractions 13–16 (left). Overall yield was improved by using more Q-Sepharose fractions when only 95% purity was sufficient (right).