PURIFICATION INVOLVING POLYMIN P FRACTIONATION OF ESCHERICHIA COLI DNA POLYMERASE III WHICH SHOWS A HIGH SEDIMENTATION CONSTANT

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1. Introduction

Polymin P, a basic polymer (polyethyleneimine) [1] has been employed to precipitate enzymes and to remove nucleic acids and ribosomes in a number of procedures of the purification of RNA polymerase [2,3] and DNA polymerase I [4,5].

During our attempts to isolate some factors which stimulate E. coli DNA polymerase III activities dependent on diadenosine 5',5''-p,p'-tetraphosphate or cyclic guanosine 3',5'-monophosphate, it was found to be convenient for the simultaneous purifications of the enzyme and protein factors from cell-free extracts to utilize polymin P so that all of these proteins will bind to subsequent DEAE-Sepharose column. To prove the occurrence of holoenzyme activity, the fractions obtained from DEAE-Sepharose column chromatography were applied to a phosphocellulose column which partially resolves β-subunit (copol III*) activity. The active fractions obtained from phosphocellulose column chromatography were further purified by glycerol gradient ultracentrifugation and the relative sedimentation constant of the fraction of the highest activity was determined. Here we show that polymin P is useful for purification of DNA polymerase III which has a high sedimentation constant (17 S) in the absence of inorganic salts.

Abbreviations: pol I, DNA polymerase I; pol II, DNA polymerase II; pol III, DNA polymerase III; pol III*, DNA polymerase III; copol III*, copolymerase III*; holoenzyme, DNA polymerase III holoenzyme; core pol III, DNA polymerase III core enzyme; DTT, dithiothreitol, ara-CTP, 1-β-D-arabinofuranosylcytosine 5'-triphosphate

2. Materials and methods

Escherichia coli K-12 cells grown to early mid-log phase were obtained from Miles Labs. E. coli P3478 (pol A1, thy-) was a gift from Dr T. Ono, The Tokyo Metropolitan Institute of Medical Science.

Polymin P was obtained from Bethesda Res. Labs. A 10% (v/v) solution titrated with concentrated HCl to pH 7.0 was prepared and clarified [2,3]. d[methyl-3H]TTP (50 Ci/mmol), d[3H]ATP (14.6 Ci/mmol), and [8-14C]ATP (61 mCi/mmol) were obtained from the Radiochemical Centre, Amersham. RNA polymerase (E. coli) was purified and assayed as in [2]. DEAE-Sepharose CL-6B and phosphocellulose (P-11) were purchased from Pharmacia Fine Chemicals and Whatman, respectively. Salmon sperm DNA was obtained from Sigma, type III, and activated as in [6,7]. Poly [d(A-T)], poly(dA) [s20,w = 6.8] and oligo(dT)10 were purchased from Miles Labs. Poly(dA): oligo(dT)10 = 1:1 (molar ratio) was prepared by annealing poly(dA) and oligo(dT)10 at 40°C for 5 min in 20 mM potassium phosphate (pH 6.8) containing 50 mM KCl. DNA concentrations are expressed as equivalents of nucleotide phosphorus.

DNA polymerase III was assayed by a minor modification of the methods in [6,7]. The standard reaction mixtures (50 µl) contained: 33 mM 4-morpholinopropane sulfonic acid–KOH (pH 7.0), 10 mM MgCl₂, 10 mM DTT, 40 µM each of dGTP, dCTP, dATP, d[3H]TTP (55 dpm/pmol), 15 nM activated salmon sperm DNA, 0.1 mg bovine serum albumin/ml, 10% ethanol (v/v) and enzyme. For the other template, poly(dA) · oligo(dT)10, activity was measured in the absence of ethanol. After incubation for 10 min at 30°C the reaction was stopped by chilling and addition of 1 ml 10% trichloroacetic acid con-
### Table 1

**Summary of purification**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Cell extract</td>
<td>1500</td>
<td><em>a</em></td>
<td><em>a</em></td>
</tr>
<tr>
<td>II. Ammonium sulfate</td>
<td>447</td>
<td>5 108 000</td>
<td>11 430</td>
</tr>
<tr>
<td>III. DEAE-Sepharose</td>
<td>127</td>
<td>3 508 000</td>
<td>27 620</td>
</tr>
<tr>
<td>IV. Phosphocellulose</td>
<td>0.4</td>
<td>481 600</td>
<td>1 204 000</td>
</tr>
<tr>
<td>V. Glycerol gradient</td>
<td>0.01</td>
<td>34 800</td>
<td>3 480 000</td>
</tr>
</tbody>
</table>

*It is difficult to get reliable and reproducible measurements of activity in fraction I due to the presence of DNA polymerase I and II.*

### 3. Results

#### 3.1. Purification procedure

The results of the purification are shown in Table 1.

**Cell extract:** Cells were lysed as in [8]. Frozen cells (300 g) were thawed in a water bath set at 4°C and suspended in 1425 ml 50 mM Tris–HCl (pH 7.5) containing 1% sodium pyrophosphate. Trichloroacetic acid precipitates were collected on Whatman GF/C filters and counted in a liquid scintillation counter. One unit is defined as the amount of enzyme catalyzing the incorporation of 1 pmol deoxynucleotide (total) into acid-insoluble material during a 10 min incubation at 37°C.

The reaction mixture (0.1 ml) for the assay of DNA polymerase I contained: 50 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 100 μM dTTP, 100 μM d[^3H]ATP (20–30 cpm/pmol), 6 nmol poly[d(A–T)], and 0.1 mg bovine serum albumin/ml. The reaction was initiated by the addition of enzyme and after 30 min incubation at 37°C, the reaction was terminated as described in the pol III assay.
37°C. The suspension was then immediately cooled in an ice bath and centrifuged for 60 min at 100,000 X g; the supernatant fluid was collected (fraction I).

**Polymin P treatment and ammonium sulfate precipitation:** The extract was placed in a beaker and a 10% solution of polymin P was added slowly with stirring to 0.35% final conc. (table 2). After further stirring for 5 min, the mixture was centrifuged for 30 min at 13,000 rev./min. The pellet was scraped into a Teflon homogenizer and resuspended in 40 ml buffer X (20 mM Tris–HCl (pH 7.2), 0.1 mM EDTA, 5 mM DTT, 20% glycerol, 1 M NaCl) with gentle stirring to avoid foaming, for 5 min. The mixture was centrifuged for 30 min at 13,000 rev./min and then solid ammonium sulfate was added to buffer X eluate to give 40% saturation. The precipitate, collected by centrifugation, was dissolved in buffer II (20 mM Tris–HCl (pH 7.2), 2 mM DTT, 20% glycerol, 0.1 mM EDTA) and dialyzed against the same buffer (fraction II).

**DEAE-Sepharose CL-6B chromatography:** Fraction II was applied to a column of DEAE-Sepharose (2.5 × 40 cm) pre-equilibrated with buffer II. It was eluted with a 21 linear gradient of buffer II to buffer II plus 0.5 M NaCl. Pol III activity emerged from the column at 0.27–0.31 M NaCl. The trace of activity which shows the characteristics of pol II was eluted from the column at 0.12–0.18 M NaCl. The pol III fractions were combined and the protein was precipitated with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in buffer III (20 mM potassium phosphate (pH 6.5), 2 mM DTT and 20% glycerol) and dialyzed against 21 same buffer (fraction III).

**Phosphocellulose chromatography:** Fraction III was applied to a column of phosphocellulose (2.5 × 36 cm) pre-equilibrated with buffer III. A linear gradient, buffer III to 0.3 M potassium phosphate (pH 6.5) containing 2 mM DTT and 20% glycerol (total vol. 800 ml) was applied and 4 ml fractions were collected. Pol III activity was eluted at 0.2–0.24 M potassium phosphate as shown in fig.1. Fractions containing pol III activity were combined, and concentrated by vacuum dialysis (collodion bag, Sartorius SM13200), and dialyzed against 20 mM Tris–HCl (pH 7.2), 10 mM DTT, 1 mM EDTA, 10% glycerol (fraction IV).

**Glycerol gradient centrifugation:** Fraction IV (0.15 ml) was layered over 5 ml of a 20–40% glycerol gradient containing 20 mM Tris–HCl (pH 7.2), 10 mM DTT and 1 mM EDTA and centrifuged for 15 h at 50,000 rev./min in a Beckman SW 65L Ti rotor at −2.5°C. Fractions of 0.2 ml were collected from the bottom of the tube. The peak fractions of pol III activity were pooled (fig.2). The pooled glycerol gradient fraction was dialyzed against 20 mM Tris–HCl (pH 7.2), 0.1 mM EDTA, 10 mM DTT and 5% glycerol and then against the same buffer containing 50% glycerol (fraction V). The enzyme was stored at −20°C where it retains 90% activity for at least 3 months.

**Preparation of β subunit:** The β subunit was partially purified as in [11] to fraction V.

3.2. Characterization of the enzyme

As shown in table 3, pol III obtained from *E. coli* K-12 wild-type, fraction V, requires a suitable template, Mg²⁺ and all 4 deoxynucleoside 5′-triphosphates like pol III from *E. coli* mutants (*polA*-, *polB*-) [6,12]. Fraction V can be distinguished from pol II and I (most of the activity of pol I appeared in the super-
Fig. 2. Glycerol gradient sedimentation profile. Centrifugation was done as described in the text. Aliquots (15 µl) of each fraction were assayed for DNA polymerase III activity (●—●). RNA polymerase (s20,w = 22 S) (X—X), β-galactosidase (s20,w = 15.9 S) (○—○), catalase (s20,w = 11.3 S) (◇—◇), and hemoglobin (s20,w = 4.5 S) (◇—◇), were used as marker proteins. RNA polymerase was assayed as in [2]. One unit of β-galactosidase, catalase and hemoglobin was defined by the method in [10].

4. Discussion

The occurrence of a few molecules of pol III in E. coli cells [15,16] makes it difficult to obtain a considerable amount of the highly purified enzyme from a relatively small quantity of the cell paste. For this and for the reasons in section 1, we tried to apply polymin P adsorption and elution procedures and glycerol gradient centrifugation in the purification starting from a relatively small cell mass. The results shown in the table 2 indicate that the pol III activity

Table 3

<table>
<thead>
<tr>
<th>Requirements for DNA polymerase III reaction</th>
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<tbody>
<tr>
<td>Components</td>
</tr>
<tr>
<td>Complete system</td>
</tr>
<tr>
<td>-dGTP, dCTP, dATP</td>
</tr>
<tr>
<td>-MgCl2</td>
</tr>
<tr>
<td>-DNA</td>
</tr>
<tr>
<td>+DNase (20 µg/ml)</td>
</tr>
<tr>
<td>+Ethanol (10%)</td>
</tr>
<tr>
<td>+KCl (0.1 M)</td>
</tr>
<tr>
<td>-DTT, +NEM (10 mM)</td>
</tr>
<tr>
<td>+ara-CTP (50 µM)</td>
</tr>
</tbody>
</table>

The enzyme used (0.015 unit) was fraction V. The reaction mixture was assayed on activated salmon sperm DNA as in section 2.

Fig. 3. ATP and β-subunit are required for the enzyme activity on poly(dA)·oligo(dT)10 template-primer. Assay conditions in the absence (A) and presence (B) of β fraction (0.2 µg) were described in section 2. The enzyme activities were determined in the presence (○—○) and absence (●—●) of 0.8 mM spermidine.
is specifically eluted from polymin P with 1 M NaCl solution. The remaining activities of pol I and II were partially removed by subsequent ammonium sulfate fractionation. Then, the traces of the activities of pol II and I were further excluded from the fractions of pol III activity by DEAE-Sepharose and phosphocellulose column chromatography, respectively. The results of fig.1 indicate the complete removal of pol I and II activities from fraction IV. In [6,12], the activity of pol III prepared from E. coli mutants (polA-; polB-) was eluted from phosphocellulose column chromatography with 0.1–0.15 M potassium phosphate (pH 6.5). The enzyme purified from E. coli P3478 (polA1) was also eluted from the column with 0.13 M potassium phosphate in our experiments (not shown). However, in these preparations from wild-type E. coli K-12, the enzyme activity eluted from a similar column at 0.20–0.24 M potassium phosphate. These results suggest differences between the structures of the 2 enzymes obtained from wild-type E. coli and polA- and polB- mutants. In [17] pol III' had a higher affinity for phosphocellulose than core pol III in the preparation from E. coli HMS-83, polA, polB mutant.

Addition of the β-subunit fraction in the presence of ATP considerably increases the activity of fraction V on the template poly(dA) - oligo(dT)10 (fig.3B). However, when the activity of fraction V was assayed in the absence of added β-subunit fraction, a relatively high ATP concentration was required for its full activation. V may be a mixture of holoenzyme and pol III*.

The high sedimentation constant (17 S) determined for the fraction of the maximal activity by glycerol gradient centrifugation (fig.2) suggests that the $M_r$ of the enzyme obtained exceeds that of β-galactosidase (540,000 $M_r$, 15.9 S), but is smaller that that of the dimer of RNA polymerase (980,000 $M_r$, 22 S). If one holoenzyme molecule is made up of 9 protein subunits expressed by $\alpha\beta\gamma\delta\epsilon\theta\tau$ [18], the $M_r$ may be 444,000. The sedimentation constant of the holoenzyme in [10] was 11 S in the presence of 0.1 M ammonium sulfate. In our experiments, 17 S enzyme activity was shifted to 11 S and 9 S in the same conditions (not shown). These results suggest some possibilities concerning the association or the subunit constitution of the enzyme molecule isolated by density gradient centrifugation (fraction V):

(i) Formation of oligomer of pol III* or holoenzyme molecules results in a high sedimentation constant in the absence of inorganic salts, 17 S; or

(ii) The enzyme molecule of fraction V consists of subunits whose number is much larger than reported [18]; or

(iii) The enzyme exists in a molecular form binding new subunit(s) besides those already known.

In the latest report concerning the constitution of holoenzyme subunits, an additional subunit, zeta(4) has been presented [19]. Further studies on the association or constitution of holoenzyme may be required for the elucidation of the above discrepancy and functions of the enzyme obtained.

References