Purification of Escherichia coli DNA Polymerase III Holoenzyme

By MILLARD G. CULL and CHARLES S. MCHENRY

Introduction

The DNA polymerase III holoenzyme is the replicative polymerase of Escherichia coli and is responsible for synthesis of the majority of the chromosome.1 The Pol III holoenzyme contains a core polymerase plus auxiliary subunits that confer its unique replicative properties, including a rapid elongation rate, high processivity, the ability to utilize a long single-stranded template coated with the single-stranded DNA binding protein, resistance to physiological levels of salt, the ability to interact with other proteins of the replicative apparatus, and the ability to coordinate the reaction through an asymmetric dimeric structure. All of these properties are critical to its unique functions. Many of these features appear to be conserved between bacterial and mammalian systems, suggesting that insight gained through studies with the Pol III holoenzyme may generalize to a variety of life forms. The replicative role of the enzyme has been established both by biochemical and genetic criteria.2-6 Holoenzyme was biochemically defined and purified using natural chromosomal assays. Only the holoenzyme form of DNA polymerase III efficiently replicates single-stranded bacteriophages in vitro in the presence of the other known replicative proteins,7-9 and only the holoenzyme functions in the replication of bacteriophage λ, plasmids, and molecules in the presence of the E. coli replicative origin, oriC.10-13 The holo-

enzyme appears to contain 10 subunits: α, τ, γ, β, δ, δ', ε, γ', χ, ψ, and θ of 129, 900; 71,000; 47,400; 40,600; 38,700; 37,000; 26,900; 16,600; 15,000 and 8,800 Da, respectively.

Tripartite Structure of DNA Polymerase III Holoenzyme

The Pol III holoenzyme is composed of three subassemblies that function to create a processive enzyme. (1) The polymerase core is composed of the polymerase subunit α, the proofreading exonuclease e, and the θ subunit of unknown function. (2) A sliding clamp, β, is required for the holoenzyme to be highly processive. X-ray crystallography14 has revealed a bracelet-like structure for the β dimer, permitting it to slide rapidly down the DNA that it presumably encircles but preventing it from readily dissociating. Protein–protein contacts between β and other components of the replicative complex tether the polymerase to the DNA, increasing its processivity. (3) A five-protein DnaX complex recognizes primer termini and closes the β bracelet around DNA. This complex remains firmly associated as part of the elongation complex between the α subunit and β. Presumably, α contacts β at a point away from the DNA15 (Fig. 1). Key subunits of all three subassemblies contact the primer in the order α, DnaX protein, and β, starting from the primer terminus (Fig. 1).

The dnaX gene of E. coli encodes two protein products, τ and γ.16,17 Both proteins contain a consensus ATP binding site near their amino

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that is used to bind and hydrolyze ATP, in concert with ψ', ψ, setting the β processivity clamp on the primer terminus. Protein γ arises by translational frameshifting, generating a 47,400-Da protein with sequences nearly identical to the amino-terminal two-thirds of τ. In addition to these interactions, τ, but not γ, can bind tightly to the DNA Pol III core, causing it to dimerize. It has been proposed that the two DnaX proteins might assemble asymmetrically, forming an asymmetric dimeric enzyme with distinct leading and lagging strand polymerases. The advantages of such an arrangement have been discussed.

**Structure of DNA Polymerase III Holoenzyme**

Insight into the structure of the DNA Pol III holoenzyme has been continually evolving. Major questions relating to the placement of the γ subunit and its associated proteins within the complex and the proposed asymmetric placement of τ relative to γ remain to be resolved. However, it is clear that the τ subunit has function in addition to dimerization of the polymerase. Like γ, it serves to bind δ, δ', χ, and ψ, and in concert with these proteins, to load β onto primers to form initiation complexes that are competent for elongation. Our working model for the structure of holoenzyme is shown in Fig. 2.

**Methods**

**Cell Growth**

Because it has not been possible to resolve DNA helicase II (uvrD gene product) chromatographically and in this way to produce DNA polymerase III holoenzyme free of this contaminant, we use an E. coli strain MGC1020 deleted in uvrD by insertion of a kanamycin-resistance cassette. E. coli K12 strain MGC1020 (W3110 lexA3, malE::Tn10, uvrD::Kn) was constructed by P1 transduction of W3110 [obtained from the American Type Culture Collection (ATCC)] to lexA3 from phage grown on strain GW2727 (constructed in the laboratory of Graham Walker) followed by P1 transduc-

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Fig. 2. Structural features of the DNA polymerase III holoenzyme. Interactions known with certainty are shown by solid lines. Lines designating direct subunit contacts extend to the specific subunit involved. Interactions between complexes extend only to the ellipse, designating an isolatable complex. Holoenzyme can be reconstituted free of γ without the loss of any detectable functions, yet native holoenzyme contains γ. Its attachment site with holoenzyme is uncertain, but it may be linked through the β subunit with which it, like τ, interacts. τ binds tightly to α, whereas γ does not. The dotted line indicates observed weak γ–τ interactions.

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10% Trichloroacetic acid (TCA)
250 mM Magnesium acetate
0.2 M Sodium pyrophosphate + 1 N HCl
0.2 M Sodium pyrophosphate
dNTP cocktail: 400 μM dATP, dGTP, dCTP, 150 μM [H³] dTTP (100 cpm/pmole)
rNTP cocktail: 5 mM ATP, GTP, CTP, UTP
1 μg/ml Rifampicin (to inhibit RNA polymerase in assays of impure enzyme only).

The polymerase assay measures DNA synthesis at 30° from a primed M13Gori template as acid-precipitated product on GF/C filters (Millipore, Cat No. 1822 024). M13Gori DNA, single-stranded binding protein (SSB), and dnaG primase (listed below) are obtained from ENZYMOCO, Inc., Denver, CO. Each 25-μl reaction contains, in order of addition:

<table>
<thead>
<tr>
<th>Assay component</th>
<th>14 μl</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme dilution buffer</td>
<td>14 μl</td>
<td>Approximately 28 mM HEPES, pH 7.5, 11.2% glycerol, 0.01% NP-40, 0.1 mg/ml BSA, 56 μM potassium glutamate, 0.3 mM DTT</td>
</tr>
<tr>
<td>250 mM Magnesium acetate</td>
<td>1 μl</td>
<td>10 mM</td>
</tr>
<tr>
<td>M13Gori DNA (OD₂₆₀ = 2)</td>
<td>2 μl</td>
<td>500 picomoles total nucleotide</td>
</tr>
<tr>
<td>SSB</td>
<td>2 μl</td>
<td>1.6 μg</td>
</tr>
<tr>
<td>dNTP cocktail</td>
<td>3 μl</td>
<td>48 mM dATP, dCTP, dGTP, 18 μM dTTP</td>
</tr>
<tr>
<td>rNTP cocktail</td>
<td>1 μl</td>
<td>200 μM rNTPs</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>0.2 μl</td>
<td>0.2 μg</td>
</tr>
<tr>
<td>DnaG primase</td>
<td>0.2 μl</td>
<td>55 units (100 ng)</td>
</tr>
</tbody>
</table>

When necessary, add EDB to bring final reaction volume to 25 μl.

Procedure. Initiate reactions by the addition of Pol III holoenzyme to the reaction mix at 0°. Transfer to a 30° bath. After 5 min, quench reactions by the addition of 2 drops 0.2 M sodium pyrophosphate and 0.5 ml 10% TCA acid, and filter through GF/C filters. Wash filters with 1 M HCl, 0.2 M sodium pyrophosphate. A final rinse should be made with ethanol. Dry filter and quantitate by scintillation counting. A unit is defined as 1 pmol of total deoxyribonucleotide incorporated/min.

Proteins are determined by Coomassie Plus Protein Assay Reagent (Pierce, Cat. No. 23236) according to the manufacturer’s instructions.

Bovine plasma γ-globulin (Bio-Rad, Cat. No. 500-0005) is used as a standard.

Cell Lysis and Ammonium Sulfate Fractionation

Stock Solutions

Buffer T + 0.1 M NaCl: 50 mM Tris–HCl, pH 7.5, 20% glycerol, 1 mM EDTA, 0.1 M NaCl
Tris–sucrose: 50 mM Tris–HCl, pH 7.5, 10% sucrose (prewarm to 42°)
Lysis solution: 50 mM Tris–HCl, pH 7.5, 10% (w/v) sucrose, 2 M NaCl, 0.3 M spermidine hydrochloride. Adjust pH to 7.5 with 10 N NaOH
2.0 M Tris base
0.5 M DTT
0.20 Ammonium sulfate backwash: Add 20 g ammonium sulfate to every 100 ml Buffer T + 0.1 M NaCl
0.17 Ammonium sulfate backwash: Add 17 g ammonium sulfate to every 100 ml Buffer T + 0.1 M NaCl.

Procedure. A key feature to this procedure is the preparation of a DNA-free lysate, a requirement for the holoenzyme to bind to the Bio-Rex 70 column and for the enzyme to remain intact in a variety of manipulations. The holoenzyme is less soluble than most proteins in ammonium sulfate. The procedure we describe permits near-quantitative precipitation of holoenzyme, and removes contaminants by backwashing with decreasing concentrations of ammonium sulfate. This purification is based on 3.6 kg of cell paste (7.2 kg of “popcorn” [frozen 1:1 (w/v) suspension of cells]). The lysis step and ammonium sulfate fractionation are typically performed in four 900-g batches. Throughout the entire holoenzyme purification procedure, DTT from a 0.5 M stock is added to buffers just before they are needed in order to minimize oxidation. All imidazole hydrochloride and Tris–HCl buffers are prepared from 0.5 M stocks adjusted to the specified pH at 25°. No additional adjustments are made. Note that ammonium sulfate concentrations are reported as the amount of ammonium sulfate added to each ml of solution, not the amount added per each ml final volume.

1. Weigh out 1.8 kg of frozen “popcorn” into a large (~5-liter) plastic bucket.
2. Pour 2475 ml of prewarmed Tris–sucrose (42°) with stirring into the frozen popcorn. The temperature of the slurry should be monitored with a thermometer and should not be allowed to exceed 4°. Stir the slurry with an overhead stirrer; avoid foaming.
3. Add 45 ml of freshly prepared 0.5 M DTT.
4. Add 225 ml lysis solution.
6. Continue moderate stirring until ice crystals have completely disappeared. Check the homogeneity of the mixture by turning off the stirrer (the ice crystals float).
7. Once a homogeneous mixture is achieved, add 0.9 g lysozyme freshly dissolved in 20 ml Tris–sucrose. Final concentration lysozyme = 0.2 mg/ml.
8. Mix thoroughly and immediately transfer to 250-ml centrifuge bottles. Leave on ice for 1 hr.
10. Return the bottles immediately to an ice bath.
11. Centrifuge at 23,000g for 1 hr at 4°.
12. Collect supernatant in a prechilled 4-liter cylinder; save a 2-ml sample for protein determinations, and record the volume. The supernatant is Fraction I. (Typical yield: 75 g protein in 3500 ml.)
13. Add 0.226 g ammonium sulfate to each ml of Fraction I slowly (over 30 min) while stirring with a magnetic stirbar. Record volume and remove 0.5 ml for assays.
14. Centrifuge at 23,000g for 30 min at 0°.
15. Using Dounce homogenizer (loose pestle), resuspend pellet in 0.125× Fraction I volume of 0.2 ammonium sulfate backwash solution + 5 mM DTT. Record volume and remove 2× 0.5-ml aliquots for assays.
16. Centrifuge at 23,000g for 45 min at 0°.
17. Using a Dounce homogenizer, resuspend pellet in 0.02× Fraction I volume of 0.17 ammonium sulfate backwash solution + 5 mm DTT. Measure volume and reserve 2× 0.5-ml aliquots to centrifuge separately and assay before dissolving the entire preparation. This will permit assessment of success of this stage of the purification and facilitate planning for subsequent steps.
18. Centrifuge at 35,000g for 30 min at 0°.
19. Pour off the supernatant, carefully seal the tube to prevent desiccation, and store the pellet (Fraction II) at −80°. (Typical yield for a 900 g prep: 0.5 g protein, 7.5 × 10⁶ units.)

**Cation-Exchange Chromatography on Bio-Rex 70**

**Stock Solutions**

DMSO buffer: 50 mM imidazole hydrochloride, pH 6.8, 20% dimethyl sulfoxide (DMSO), 10% glycerol, 5 mM DTT

DMSO buffer + 0.1 M NaCl: 50 mM imidazole hydrochloride, pH 6.8, 0.1 M NaCl, 20% DMSO, 10% glycerol, 5 mM DTT

DMSO buffer + 0.2 M NaCl: 50 mM imidazole hydrochloride, pH 6.8, 0.2 M NaCl, 20% DMSO, 10% glycerol, 5 mM DTT

Bio-Rex 70 elution buffer: 50 mM imidazole hydrochloride, pH 6.8, 0.5 M NaCl, 30% glycerol, 1 mM EDTA, 5 mM DTT.

**Procedure.** Cation exchange provides a powerful chromatographic step for all forms of DNA Pol III. However, strong cation exchangers cause the β subunit to dissociate. The relatively weak but high-capacity cation exchanger Bio-Rex 70 permits the holoenzyme to remain intact. Its high capacity is also important since it permits the holoenzyme to be kept concentrated, minimizing losses due to its dilution sensitivity. Holoenzyme is most stable at relatively high concentrations of salt. The presence of DMSO in the loading buffers permits the holoenzyme to bind to the column at higher concentrations than is otherwise possible. The decreasing DMSO concentrations in the gradient permit elution of the enzyme.

1. Pour a Bio-Rex 70 (100 to 200 mesh) column and equilibrate it with DMSO buffer without DTT. After column equilibration run at least two more column volumes of DMSO buffer + 5 mM DTT through the column. The correct column size can be estimated by using 1 ml resin for every 20 mg protein in Fraction II. Bio-Rex used for the first time should be washed in both acid and base before use. Batch wash the Bio-Rex with 0.5 M imidazole hydrochloride, pH 6.8, before pouring the column. Used Bio-Rex can be recycled by washing with 2 M NaCl. Used resin gives higher yields. Bio-Rex 70 has an exceedingly high capacity and takes a long time to equilibrate. Carefully check the pH and conductivity of the column effluent.

2. Thaw Fraction II pellets on ice, centrifuge (34,800g, 0°, 10 min) and remove any remaining supernatant. Dissolve Fraction II pellets in ice-cold DMSO buffer + 0.1 M NaCl + 5 mM DTT. Use 1 ml of buffer for every 25 g of cells to dissolve the Fraction II pellets (144 ml for 3.6 kg preparation). Dounce-homogenize the resuspended pellets to achieve a homogeneous mixture. Save a 0.5-ml aliquot for assays and conductivity determination.

3. Centrifuge suspension (34,800g, 0°, 1 hr) to clarify. During centrifugation, test the conductivity of the sample from the previous step. If the sample conductivity is not in the range of the conductivity of DMSO buffer + 0.1 M NaCl to DMSO buffer + 0.2 M NaCl, dilute the clarified sample with DMSO buffer + 5 mM DTT to bring it within this range. Typically this dilution requires 2 volumes of DMSO buffer. (Note: Unless stated otherwise, all conductivities in this preparation are determined on 1/100 dilutions.)
4. Apply the clarified Fraction II to the Bio-Rex 70 column at a rate of 2 column volumes/hr. Wash the column with 1 column volume of DMSO buffer + 0.2 M NaCl. The majority of the contaminating protein flows through the column (Fig. 3A).

5. Elute the enzyme with a 4 column volume gradient at a flow rate of 2 column volumes/hr. Gradient starting buffer: DMSO buffer + 0.2 M NaCl. Collect ~60 fractions, save 100 μl of each fraction for assays, and immediately add an equal volume of saturated ammonium sulfate to the fractions. The enzyme is unstable during this chromatographic step, but becomes stable once ammonium sulfate is added.

6. Assay samples for activity and combine all fractions that contain at least 50% of the activity of the peak fraction. Centrifuge the ammonium sulfate precipitates repeatedly (34,800g, 0°C, 1 hr) in two 34-ml centrifuge tubes, so that pellets can be dissolved in a minimal volume in the next step. Store pellets (Fraction III) on ice. Record volume of pooled samples, save a sample for assays, and centrifuge it separately [Typical yield: 45 mg protein, 1.3 × 10^7 units (Table I).]

**Hydrophobic Interaction Chromatography on Valyl-Sepharose**

Hydrophobic interaction chromatography permits purification of holoenzyme in high concentrations of stabilizing salt. This step was developed before the commercial availability of hydrophobic resins. We have not tested substitutes and continue to make our own resin. Preparation is relatively convenient, inexpensive, and does not need to be performed often since the resin can be recycled if properly handled.
TABLE I
PURIFICATION OF DNA POLYMERASE III HOLOENZYME

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Units ($\times 10^{-6}$)</th>
<th>Protein (mg)</th>
<th>Volume (ml)</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>30</td>
<td>1860</td>
<td>390</td>
<td>16,100</td>
</tr>
<tr>
<td>III</td>
<td>13</td>
<td>45</td>
<td>85</td>
<td>290,000</td>
</tr>
<tr>
<td>IV</td>
<td>3.2</td>
<td>5.8</td>
<td>0.4</td>
<td>550,000</td>
</tr>
<tr>
<td>V</td>
<td>1.7</td>
<td>1.5</td>
<td>6.0</td>
<td>1,160,000</td>
</tr>
</tbody>
</table>

* 3.4 kg cells.

Stock Solutions

Buffer 1 + 0.1 M NaCl: 50 mM imidazole hydrochloride, pH 6.8, 0.1 M NaCl, 20% glycerol, 1 mM EDTA, 5 mM DTT

2.0 M Ammonium sulfate buffer: 50 mM imidazole hydrochloride, pH 6.8, 10% glycerol, 2.0 M ammonium sulfate, 5 mM DTT

1.2 M Ammonium sulfate buffer: 50 mM imidazole hydrochloride, pH 6.8, 10% glycerol, 1.2 M ammonium sulfate, 5 mM DTT

0.4 M Ammonium sulfate buffer: 50 mM imidazole hydrochloride, pH 6.8, 20% glycerol, 0.4 M ammonium sulfate, 5 mM DTT

Resin Preparation. Valyl-Sepharose is prepared by cyanogen bromide activation of Sepharose 4B as previously described with minor modifications. After activation of the beads, an equal volume of 0.2 M L-valine in 0.2 M NaHCO₃ is added to the beads. This slurry is allowed to incubate with shaking at 4°C for 20 hr. After coupling, the beads are washed with 20 volumes each 0.1 M sodium acetate plus 0.5 M NaCl (pH 4 with glacial acetic acid), followed by 0.1 M NaHCO₃, pH 9.5, wash, and a final wash with 0.5 M NaCl. The resin is stored in 0.5 M NaCl. The resin has about 12 µmol valine/ml as determined by acid hydrolysis, filtration, and amino acid analysis.

Procedure

1. Pour a valyl-Sepharose column and equilibrate it in 1.2 M ammonium sulfate buffer. The correct column size can be estimated by using 1 ml resin for every 0.78 mg protein present in Fraction III.
2. Dissolve Fraction III pellets in Buffer I + 0.1 M NaCl. Bring the final concentration to 1 mg/ml.
3. Separately equilibrate 10% of the volume of the valyl-Sepharose column and add the dissolved Fraction III to it (first record volume and save a sample for assays). Slowly (over 15 min) add 1.5× the volume of the redissolved Fraction III of 2 M ammonium sulfate buffer with vortexing. This step will allow a uniform coating of protein on the beads rather than precipitation of the proteins onto the top of the column. Ammonium sulfate must be added slowly, especially the final amount, to permit uniform coating of the beads.
4. Apply the coated beads and the accompanying solution to the column. Wash the column with 1 column volume of 1.2 M ammonium sulfate buffer.
5. Run a 10 column volume gradient at 0.8 column volume/hr. Gradient starting buffer: 1.2 M ammonium sulfate buffer. Eluting buffer: 0.4 M ammonium sulfate buffer. Collect 60 fractions and assay the fractions for activity. Holoenzyme elutes approximately halfway through the gradient (Fig. 3B).
6. Pool fractions that have at least 50% of the activity of the peak tube and, after recording the volume and saving an aliquot for assays, add 0.262 g ammonium sulfate/ml of pooled fractions and stir overnight on ice. Centrifuge in one 34-ml tube repeatedly to permit resuspension of the resulting pellets in a small volume. This is necessary to ensure that a concentrated sample can be applied to the DEAE-Sephadex column in the next step. Store the pellets (Fraction IV) on ice. [Typical yield: 5.8 mg protein, 3.2 × 10⁶ units (Table I).]

Ion-Filtration Chromatography on DEAE-Sephadex

Poor yields result when holoenzyme is diluted or dialyzed and then bound to standard anion-exchange columns, presumably due to its sensitivity to dilution and low salt. The ion-filtration technique improves the yields obtained for holoenzyme several fold. The method was developed by determining a salt concentration that permitted interaction and retardation of holoenzyme by the matrix without binding so tightly that it required higher salt for elution. Contaminants that do not interact with the column elute in about one-third column volume, just as in gel filtration for excluded proteins. Proteins that interact strongly elute in 1 column volume where the high salt wash elutes. Because of this delicate balance, buffers must be prepared precisely.

Stock Solutions

Buffer 1 + 120 mM NaCl: 50 mM imidazole hydrochloride, pH 6.8, 120 mM NaCl, 20% glycerol, 1 mM EDTA, 5 mM DTT


By Marjorie H. Barnes and Neal C. Brown

Introduction

Bacteria possess three classes of DNA polymerase, designated I, II, and III; of these, DNA polymerase III (Pol III) is essential for replicative DNA