Cloning and Simplified Purification of *Escherichia coli* DNA Gyrase A and B Proteins*

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We have transferred the *Escherichia coli* gyrA and gyrB genes onto plasmids that allow the overproduction of the DNA gyrase A and B proteins and have designed relatively simple purification procedures for both proteins. The pure proteins are obtained in good yield; from 2 liters of culture (12 g of cells), one can recover 25 mg of GyrA or 3 mg of GyrB protein.

DNA gyrase, the enzyme responsible for bacterial DNA supercoiling (1, 2), is made up of two subunits, the products of the gyrA and gyrB genes (2-4). The enzyme has been purified from *Escherichia coli* either as the active complex (5) or as the separate GyrA and GyrB proteins from which the supercoiling activity is reconstituted by mixing (3, 6-8). Although moderate amounts of DNA gyrase can be obtained from wild-type *E. coli* (a recent method yields about 1 mg of each subunit from 100 g of cells (8)), many experimental studies on the structure of the enzyme and its complex with DNA and on the enzymatic reactions would be much easier with larger supplies of purified enzyme. For these reasons, we have moved the gyrA and gyrB genes onto high-copy-number plasmids and have developed simplified purification procedures for both the GyrA and ByrB proteins. The plasmids are also useful in studies of the regulation of DNA gyrase synthesis (9).

**MATERIALS AND METHODS**

Polymin P, obtained from BASF (Rhein, Germany), was neutralized to pH 7.9 with HCl before use. DEAE-Sepharose CL-6B was from Pharmacia, heparin-agarose from Bethesda Research Laboratories, hydroxylapatite (Bio-Gel HTP) from Bio-Rad Laboratories, leucine-agarose from Sigma, and bovine serum albumin from Calbi-ochem-Behring. Valine-Sepharose was made by the method of Rimerman and Hatfield (10). TGED buffer is 0.05 M Tris-HCl (pH 7.5), 10% (w/v) glycerol, 1 mM Na,EDTA, 5 mM dithiothreitol. TGED-2 is the same buffer with the EDTA concentration reduced to 0.2 mM. TGED-3 is TGED-2 buffer with the Tris-HCl (pH 7.5) concentration reduced to 0.02 M.

**Assay of DNA Gyrase Supercoiling Activity**

Fractions were assayed for GyrA or GyrB activity in the presence of an excess of the other subunit. When GyrB activity was assayed, 50-100 units of GyrA were added; when GyrA activity was assayed, 25 units of GyrB were added. Assay conditions were as described (5), except that the concentration of MgCl₂ was reduced to 4 mM. Enzyme fractions were diluted as previously described (5). One unit of DNA supercoiling activity is defined as converting half of the DNA in the standard assay to a fully supercoiled species in agarose gel electrophoresis. In the normal assay, diluted GyrA and GyrB proteins are added separately to a reaction mixture which contains all the other components. In a variant assay procedure, which gives a several-fold higher specific activity for GyrB (see "Results" and Table I), the GyrA and GyrB proteins are preincubated together at high concentration (0.05-0.25 mg/ml of each protein) in TGED-2 buffer containing 50% (w/v) glycerol and 70 mM KCl for 30 min at 20°C. The enzyme is then diluted as usual and the assay is started promptly.

**Cloning of gyrA and gyrB Genes onto Plasmids**

The plasmid vector for cloning gyrA was pCK16 (11), composed essentially of the large EcoRI-BamHI fragment of pBR322 and the N-cl-O-P region of phage λ. A BamHI fragment (approximately 11 kilobases) containing the gyrA gene was isolated from λnanA (2) and was inserted at the BamHI site of pKC16. Transformants in *E. coli* strain RW1053 recA Δ(gal attλ bio) were screened for overproduction of GyrA protein. Strain N4186 = RW1053 (pMK90) was used for the enzyme purification described below.

To clone gyrB, we took advantage of the fact that the genome region near gyrB contains genes (e.g. dnaN and dnaA) whose presence on high-copy-number plasmids could lead to poor growth of the host cells. (In the present case, this precaution may have been unnecessary; see below.) We therefore constructed a composite plasmid vector that could be maintained at either high- or low-copy number by replication from either the pBR322 origin or from the F factor origin. In a polA host strain, the pBR322 origin is inactive (12) and the plasmid copy number drops to that of the F factor (about three copies/cell). To construct this vector, pMM121, we joined the large EcoRI fragment of pKC16 to the EcoRI-5 fragment of F(Δ-15) (13); the small BamHI fragment of EcoRI-5 was deleted in the process. Due to the loss of the small EcoRI fragment of pKC16 that contained phage λ replication functions, pMM121 cannot replicate from the λ origin. Plasmid pMM121 was linearized by partial digestion with BamHI nuclease. It was then ligated to a BamHI fragment (approximately 10 kilobases) which had been isolated from λnanA (14). Ampicillin-resistant transformants were selected first in *E. coli* N1069 polA and screened for the proper orientation of the inserted fragment relative to the P₈ promoter of the vector. The DNA of one such transformant plasmid, pMM115, was then used to transform strain RW1053 (polA). Transformants in the polA and pol₈ strains grew at similar rates; the plasmid was therefore maintained in RW1053.

To reduce the size of the cloned segment, a HindIII fragment containing the gyrB, recF, dnaN, dnaA, and λN genes was trimmed with nuclease Bal31 and then cut with BamHI and inserted at the BamHI site of pKC16. After transformation of RW1053 and selection for ampicillin resistance, the resulting clones were screened for the orientation of the inserted fragment and for high production of GyrB protein. One such transformant, MK47, clearly produced more GyrB than RW1053 (pMM115) and was therefore used as a source of GyrB in the purification procedure described below. The plasmid contained in this strain is designated pMK47.

**Growth and Lysis of Cells**

Cells were grown in a 100- or 300-liter fermentor in a medium containing, per liter, 10 g of Bacto-tryptone (Difco), 5 g of yeast extract (Difco), 10 g of NaCl, 5 g of glucose, 5 ml of 1 M potassium phosphate (pH 7.0), and 1 mg of biotin. The last three ingredients were added separately after autoclaving. The culture was aerated at 32°C until A₆₉₀ reached 0.6. The temperature was raised to 42°C for 15 min and then lowered to 37°C; aeration was continued throughout.

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(Received for publication, February 1, 1984)
The pH of the culture was continuously adjusted to 7.0 by adding 5 M KOH. At $A_{600} = 4.0$, the culture was chilled and the cells were collected by centrifugation (about 600 g of packed cells from a 100-liter culture).

The cells were resuspended in 0.05 M Tris-HCl (pH 7.5), 10% sucrose (100 g of cells) at low speed in a Waring blender, frozen in 40-ml aliquots in liquid nitrogen, and stored at -70 °C.

Cell lysates were prepared by the action of lysozyme and Brij-58, as described (15). All the purification steps were carried out at 0–4 °C. All centrifugations were at 10,000 × g for 15–20 min.

**Purification of DNA Gyrase A Protein**

*Polymin P Fractionation*—25 ml of a crude extract of strain N4186 (fraction 1; protein concentration of 21.4 mg/ml) was thawed, diluted to a final protein concentration of 8 mg/ml with 0.05 M Tris-HCl (pH 7.5), 10% sucrose, and brought to 0.2 M NaCl with 4 M NaCl. To the resulting 62 ml of diluted extract, 4.3 ml (0.07 volume) of 5% Polymin P were added over 10 min with stirring, and stirring was continued for 15 min. After centrifugation, the bottles were drained and the pellets were resuspended in 25 ml of 0.05 M Tris-HCl (pH 7.5), 1 mM Na3EDTA, 1 mM dithiothreitol with the use of a stirring rod. After further stirring for 15 min, the suspension was centrifuged, the supernatant was discarded, and the pellets were resuspended in 25 ml of 1 M NaCl, 0.05 M Tris-HCl (pH 7.5), 1 mM Na3EDTA, 1 mM dithiothreitol. After stirring for 15 min, the suspension was centrifuged and the supernatant solution was collected. Solid ammonium sulfate (0.31 g/g of supernatant) was added, and the suspension was stirred for 15 min. The precipitate was collected by centrifugation and resuspended in 4 ml of 0.1 M KCl, 0.05 M Tris-HCl (pH 7.5), 1 mM Na3EDTA, 1 mM dithiothreitol (fraction 2; 6.0 ml, 90 mg of protein).

**DEAE-Sepharose Chromatography**—Fraction 2 was diluted with 4 liters of TGED buffer, centrifuged to remove a precipitate that formed during dialysis, and then diluted with 2 volumes of TGED buffer. The sample was loaded onto a DEAE-Sepharose column (bed volume of 3 ml) of hydroxylapatite, previously equilibrated with TGED buffer containing 0.02-0.5 M potassium phosphate (pH 7.5) in TGED. A 3.75-m1 portion of fraction 2 was loaded onto a 1 ml column. After washing with 8 ml of the equilibration buffer, the protein was eluted with a 240-ml linear gradient of 0.025-0.3 M NaCl in TGED-2 buffer. Fractions with GyrB activity were eluted around 0.1 M NaCl (fraction 4; 32 ml, 21.2 mg of protein).

**Hydroxyapatite Chromatography**—Fraction 4 was loaded onto a column (bed volume of 3 ml) of hydroxyapatite, previously equilibrated with 0.02 M potassium phosphate (pH 6.8), 5 mM dithiothreitol, 10% (w/v) glycerol. After washing with 25 ml of TGED buffer, the column was developed with a 90-ml gradient of 0.02-0.5 M potassium phosphate (pH 7.5) containing 5 mM dithiothreitol, 10% (w/v) glycerol. Active fractions were eluted around 0.23 M potassium phosphate (fraction 5; 16.3 ml, 6.5 mg of protein).

**Leucine-Agarose Chromatography**—A 2-ml column of leucine-agarose was washed with TGED buffer and equilibrated with 1.5 M potassium phosphate (pH 7.5) in TGED. A 3.75-ml portion of fraction 5 was eluted in small aliquots with 3 volumes of 2 M potassium phosphate (pH 7.5), 1 mM Na3EDTA, 5 mM dithiothreitol and loaded onto the column. The column was washed with 8 ml of the equilibration buffer, and the protein was eluted with a 60-ml linear gradient of 1.5–0.5 M potassium phosphate (pH 7.5) in TGED buffer. GyrB activity was eluted around 1.15 M potassium phosphate (fraction 6; 13 ml, 1.6 mg of protein).

Fractions 1-6 were all stable at -70 °C for at least 1 year.

**RESULTS AND DISCUSSION**

Table I summarizes the purification procedures. The two activities are purified with an overall recovery of about 50% for GyrA and 40% for GyrB. Both protein preparations are nearly homogeneous when displayed in sodium dodecyl sulfate-polyacrylamide electrophoresis (Fig. 1). Very minor contaminants (<2% total) that could be seen in the original gel are not visible on the photographic print.

The strains that carry gyrA or gyrB plasmids, combined with the purification method described above, are a useful source of both DNA gyrase proteins. From 12 g of packed cells (the yield of about 2 liters of culture), one can recover more than 25 mg of purified GyrA protein or 3 mg of GyrB protein. The higher yield of GyrA protein reflects its higher source of both DNA gyrase proteins. From 12 g of packed cells (the yield of about 2 liters of culture), one can recover more than 25 mg of purified GyrA protein or 3 mg of GyrB protein. The higher yield of GyrA protein reflects its higher

| Step | Volume | Total Protein | Specific Activity | Total Activity |
|------|--------|---------------|------------------|---------------|
|      | ml     | mg            | units/mg (mg)    | units         |
|**GyrA Protein** |        |               |                  |               |
| 1. Extract | 23.0 | 493 | 1.4 $\times$ 10$^9$ | 7 $\times$ 10$^9$ |
| 2. Polyamin-ammonium sulfate | 6.0 | 90.0 | 5 $\times$ 10$^9$ | 5 $\times$ 10$^9$ |
| 3. DEAE-Sepharose | 22.0 | 62.4 | 8 $\times$ 10$^9$ | 5 $\times$ 10$^9$ |
| 4. Valine-Sepharose | 187.0 | 39.0 | 8 $\times$ 10$^9$ | 4 $\times$ 10$^9$ |
|**GyrB Protein** |        |               |                  |               |
| 1. Extract | 33.5 | 1300 | 1.0 $\times$ 10$^9$ | 3.3 $\times$ 10$^9$ |
| 2. Streptomycin-ammonium sulfate | 15.0 | 670 | 1.8 $\times$ 10$^9$ | 1.2 $\times$ 10$^9$ |
| 3. Heparin-agarose | 20.8 | 110 | 8 $\times$ 10$^9$ | 8 $\times$ 10$^9$ |
| 4. DEAE-Sepharose | 32.0 | 21.2 | 3 $\times$ 10$^9$ | 6 $\times$ 10$^9$ |
| 5. Hydroxylysinate | 16.3 | 8.6 | 6 $\times$ 10$^9$ | 6 $\times$ 10$^9$ |
| 6. Leucine-agarose | 65.1 | 7.0 | 7 $\times$ 10$^9$ | 5 $\times$ 10$^9$ |

* These steps were run using an aliquot of the preceding fraction, as described under “Materials and Methods.” The numbers in this table have been adjusted accordingly.

The values in parentheses refer to the modified assay in which the GyrA and GyrB proteins are preincubated together at high concentration; the specific activity of each protein is determined with an excess (in units) of the other (see “Materials and Methods”).

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content in the crude extract, where at least 10% of the total protein is seen to be GyrA (Fig. 1). Even the lower content of GyrB reflects at least a 20-fold overproduction by MK47 compared to a normal E. coli strain.

Neither protein preparation had any detectable DNA supercoiling activity in the absence of the complementing protein (assayed with 100 units), indicating the absence of cross-contamination of the A and B subunits. Both protein preparations assayed singly were also free of contaminating topoisomerase and endonuclease activity under the gyrase assay conditions (none detected with 100 units of GyrA or GyrB protein). The ATPase activity of the gyrase complex was inhibited more than 95% by novobiocin in the absence of magnesium ion, ATP, DNA, or any combination of them to the preincubation. When GyrA is in molar excess, the increase of the specific activity of GyrB is independent of molar ratio; when GyrB is in molar excess, there is no increase of GyrA activity.

When assayed in this way, our preparation of GyrB has a specific activity 2.5-fold less than GyrA. As a practical measure, the preincubation makes it possible to use the two subunits at comparable molar concentrations without a great sacrifice in activity. The specific activities of the subunits measured in this way are at least as high as those previously reported for GyrA (8), GyrB (7, 8), or the purified complex (5) when adjusted for different definitions of units. The mechanism of the activation is still obscure. It is reversed after dilution of the enzyme into the assay medium if the tubes are kept at 0 °C for 30 min before being transferred to 25 °C to start the supercoiling reaction, a result which again implicates the state of aggregation of the enzyme. Further studies on the process are being carried out.

Acknowledgments — We thank N. Cozzarelli and K. von Meyenburg for sending & phages containing the gyrA and gyrB genes, Nancy Nosslin for advice and help in preparing valine-Sepharose, Joseph Shiloach for growing large batches of cells, and Anthony Maxwell for carrying out the ATPase assays.

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FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of successive fractions in the purification of gyrase A and B proteins. The lanes are identified by the fraction numbers (see text and Table I).