Purification and properties of DNA gyrase from *Vibrio cholerae*

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The DNA gyrase was purified from *Vibrio cholerae* strain 569B. It appeared to be composed of two subunits of M_r 120 000 and 97 000 and had a great deal of similarity to the *Escherichia coli* gyrase. Unlike the *E. coli* enzyme, however, it could neither relax supercoiled DNA nor induce a cleavage of double-stranded DNA, under experimental conditions in which *E. coli* gyrase could do so.

INTRODUCTION

Expression of a number of genes in bacteria is modulated by the state of negative superhelicity of its DNA. Negative superhelical conformation of the *Escherichia coli* DNA is actively maintained by the enzyme DNA gyrase (Cozzarelli, 1977). This enzyme, which catalyses the supercoiling of relaxed closed circular DNA in the presence of ATP (Gellert *et al.*, 1977), is considered to be an essential enzyme and is assumed to be present in all bacteria (Yang & Ames, 1988). To date, however, it has been purified from only a few bacterial species (Gellert *et al.*, 1976; Liu & Wang, 1978; Sugino & Bott, 1980; Orr & Staudenbauer, 1982; Miller & Scurlock, 1983; Takahata & Nishino, 1988). In the present paper we report the isolation and characterization of the DNA gyrase from *Vibrio cholerae*, the intestinal pathogen responsible for the disease cholera.

MATERIALS AND METHODS

Growth of bacteria

V. cholerae 569B cells were grown (Mitra *et al.*, 1986; Chakravarti & Ghosh, 1987) in LB medium and collected by centrifugation at 4 °C in a Sorval GSA rotor at 8000 rev./min for 10 min. The pellet was washed once with 50 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA and 50 mM-NaCl and then stored frozen at –80 °C until further use.

Enzyme purification

Frozen cells (12.5 g) were thawed in ice in buffer A (50 mM-Tris/HCl buffer, pH 7.4, containing 1 mM-EDTA and 0.1 mM-phenylmethanesulphonyl fluoride) containing 10% (w/v) sucrose. The cells were then lysed by adding dithiothreitol, EDTA, KCl, lysozyme and Brij-58 solutions to a final concentration of 2.5 mM, 10 mM, 50 mM, 1 mg/ml and 0.2% respectively, followed by gentle mixing and incubation at 0 °C for 30 min. After the incubation period was over, a cleared lysate was made from the above, by centrifuging the mixture at 12 000 rev./min in a Sorval SS-34 rotor for 90 min at 0 °C.

DNA-bound proteins were precipitated from the lysate by the dropwise addition of neutralized Polymin P at 0 °C to a final concentration of 0.34%. The precipitate was collected by centrifugation (8000 rev./min in a Sorval SS-34 rotor for 10 min at 0 °C) and was washed once with buffer B [buffer A containing 5 mM-dithiothreitol and 10% (w/v) glycerol]. The pellet was extracted with 1 M-KCl in buffer B and centrifuged as before. Saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 7.0, was next added to the supernatant to give 55% saturation. Precipitated proteins collected by centrifugation were dissolved in buffer C (buffer B

containing 50 mM-KCl) and then dialysed against the same buffer for 3 h at 0 °C. The dialysis residue thus obtained was freed from any precipitate formed during dialysis by centrifugation, and the supernatant (fraction II) was applied to an 8 ml DEAE-Sephacel (Pharmacia) column, pre-equilibrated with buffer C.

The column was washed three times with buffer C and then eluted with a 50 ml linear gradient of 0.25–0.6 M-KCl in buffer B. DNA gyrase activity came out as a broad peak in the range of 0.25–0.35 M-KCl. Active fractions were pooled and proteins were precipitated by 50% saturation with $(\text{NH}_4)_2\text{SO}_4$. The pellet was suspended in 0.6 ml of buffer D (buffer C without phenylmethanesulphonyl fluoride and containing 100 mM-KCl instead of 50 mM) and dialysed against the same buffer for 3 h at 0 °C. The dialysis residue thus obtained (fraction III) was then applied to a Sephacryl S-300 (Pharmacia) gel-filtration column (79 cm × 1.1 cm) and the column was eluted with buffer C. Active fractions were pooled, dialysed against buffer E [25 mM-Tris/HCl buffer, pH 7.4, containing 50 mM-KCl, 2 mM-dithiothreitol and 10% (w/v) glycerol] (fraction IV) and then applied to a 3.5 ml heparin–Sephacel (Pharmacia) column previously equilibrated with the same buffer. The column was washed with 20 ml of buffer E and the eluted with a 20 ml linear gradient of 0.05–0.6 M-KCl in 25 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-dithiothreitol and 10% (w/v) glycerol. Activity came out as a sharp peak in two fractions between 0.35 M- and 0.45 M-KCl. The fractions were pooled (fraction V) and dialysed against the storage buffer [50 mM-Tris/HCl buffer, pH 7.4, containing 2 mM-dithiothreitol, 1 mM-EDTA and 50% (w/v) glycerol] for 20 h with two changes at 0 °C. The enzyme purified in this way was stable for 2 months at –20 °C.

Determination of protein

In each step of purification the protein content of the fractions was measured by the method of Bradford (1976), with BSA as standard.

Electrophoretic analysis

SDS/PAGE was performed in 10% polyacrylamide slab gels as described by Laemmli (1970). After electrophoresis, the gel was silver-stained by using the protocol of Schoenle *et al.* (1984).

Enzyme assay

The enzyme was assayed essentially as described by Mizuuchi *et al.* (1978) with minor modifications. The reaction mixture (30 μ l) contained 35 mM-Tris/HCl buffer, pH 7.5, 10 mM-MgCl₂, 2 mM-spermidine hydrochloride, 25 mM-KCl, 5 mM-dithiothreitol, 5 mM-ATP, 5% (w/v) glycerol, 0.36 mg of BSA and

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Table 1. Purification of *V. cholerae* DNA gyrase

See the text for further details. Abbreviation: N.D., not determined.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Extract (I)	327	N.D.	N.D.
Polymin P precipitate (II)	25.9	N.D.	N.D.
DEAE-Sephacel (III)	13.8	16666	1208
Sephacryl S-300 (IV)	3.8	10000	2632
Heparin-Sepharose (V)	0.141	4000	28369

0.25 μ g of relaxed covalently closed circular pBR322 or pUC19 DNA, prepared essentially by the method of Staudenbauer & Orr (1981). Enzyme was diluted into 50 mM-Tris/HCl buffer, pH 7.5, containing 20 mM-KCl, 1 mM-EDTA, 2 mM-dithiothreitol, 50% (w/v) glycerol and 50 μ g of BSA/ml. The reaction mixture was incubated at 30 °C for 30 min. Reaction was terminated by the addition of 0.2 vol. of a mixture containing 5% (w/v) SDS, 25% (w/v) glycerol and 250 μ g of Bromophenol Blue/ml and run on a 1% agarose gel in Tris/borate/EDTA buffer (10.8 g of Tris base, 5.5 g of boric acid and 0.93 g of Na₂EDTA per litre). One unit of enzyme is defined as the amount of enzyme required to convert 50% of the relaxed pBR322 or pUC19 DNA into supercoiled form under the standard assay conditions described above.

Determination of M_r

The M_r of the native fraction V enzyme was determined by gel filtration through a Sephacryl S-300 (Pharmacia) column (79 cm $\times$ 1.1 cm) equilibrated with buffer C. The column was calibrated with thyroglobulin (M_r 668 000), ferritin (M_r 440 000), catalase (M_r 232 000) and aldolase (M_r 154 000), all from Sigma Chemical Co. Protein composition of the enzyme was examined by SDS/PAGE according to the procedure of Laemmli (1970). A kit of marker proteins with M_r values ranging from 44 000 to 116 000 (Sigma Chemical Co.) was used for calibration: β -galactosidase (M_r 116 000), phosphorylase *b* (M_r 97 400), BSA (M_r 66 000) and egg albumin (M_r 44 000).

RESULTS AND DISCUSSION

The results of a typical purification of the DNA gyrase from *V. cholerae* are summarized in Table 1. This purification procedure yielded an enzyme preparation of high specific activity. Difficulty in accurately determining the gyrase activity in crude or semi-crude preparations prevented the determination of the specific activity of the enzyme in steps I and II of the purification in Table 1 and thus prevented an accurate determination of the extent of purification. Prepared this way a typical batch of *V. cholerae* DNA gyrase had a specific activity of 28 000 units/mg of protein.

Gel filtration of the fraction V enzyme on a calibrated Sephacryl S-300 column gave an M_r of 420 000 (results not shown). SDS/PAGE of fraction V enzyme revealed the presence of two polypeptides of M_r 120 000 and 97 000 in similar quantities (Fig. 1). Other bands, if present, were undetectable. These data, taken together, seemed to indicate that the DNA gyrase from *V. cholerae* is probably made up of four subunits of two different kinds and could have composition of the type A₂B₂.

V. cholerae DNA gyrase was found to be able to catalyse the supercoiling of relaxed pBR322 or pUC19 DNA with equal efficiency under the standard assay conditions described above

Fig. 1. SDS/PAGE of the fraction V enzyme

A 2 μ g portion of protein was applied to the SDS/10%-polyacrylamide gel. Positions of the M_r markers run on the same gel are indicated.

(Fig. 2). However, the supercoiling reaction could not take place if ATP were omitted from the reaction mixture (Fig. 2b). The optimal concentration of ATP required was found to be in the range 1–5 mM. The apparent K_m for ATP was determined to be 3.1×10^{-4} M (results not shown). No other nucleoside triphosphate could substitute for ATP. The enzyme also showed no activity in the absence of Mg²⁺ (Fig. 2c). However, 10 mM-Mg²⁺ in the assay mixture was more than enough to activate the enzyme, with the minimal concentration for the optimal reaction being 5 mM. Both Mn²⁺ and Ca²⁺ could substitute for Mg²⁺, albeit weakly. If KCl was omitted from the standard assay mixture, the ability of the enzyme to catalyse the supercoiling reaction diminished considerably. The optimal concentration required was found to be in the range 25–30 mM (results not shown).

The sensitivity of *V. cholerae* DNA gyrase to known inhibitors of *E. coli* DNA gyrase, namely nalidixic acid, oxolinic acid and novobiocin, was tested. Enzyme assay was performed in the standard reaction mixture described above with 40 ng (1.2 units) of enzyme in the presence of different concentrations of the drugs. *V. cholerae* enzyme was found to be very sensitive to novobiocin, with the supercoiling reaction being completely inhibited at a drug concentration as low as 1 μ g/ml. In contrast,

Fig. 2. Assay, reaction requirements and drug-sensitivity of *V. cholerae* DNA gyrase

(a) Lane 1, relaxed pUC19 DNA; lane 2, supercoiled pUC19 DNA; lane 3, relaxed pUC19 DNA incubated with 40 ng (1.2 units) of purified DNA gyrase under standard reaction conditions. (b) Lane 1, relaxed pUC19 DNA incubated with purified gyrase as in (a), lane 3; lane 2, same as in lane 1 but with ATP omitted. (c) Lane 1, relaxed pBR322 DNA incubated with DNA gyrase as in (a), lane 3; lane 2, as in lane 1 but with Mg^{2+} omitted. (d) Relaxed pBR322 DNA incubated with *V. cholerae* DNA gyrase as in (c), lane 1, but with the following additions: lane 1, oxolinic acid (20 μ g/ml); lane 2, nalidixic acid (200 μ g/ml); lane 3, novobiocin (1 μ g/ml); lane 4, none. This Figure is a composite from experiments performed on different days. In each of the experiments described here and in Figs. 3 and 4, 40 μ g of enzyme (1.2 units) was used per reaction, unless otherwise specified. Positions of the various forms of the plasmid DNA are indicated by bars: top, relaxed; bottom, supercoiled.

Fig. 3. Inability of *V. cholerae* DNA gyrase to induce cleavage of double-stranded DNA in the presence of oxolinic acid

Intracellularly supercoiled pBR322 DNA was treated under a variety of conditions (see the text for further details and the legend to Fig. 2). Lane 1, pBR322 DNA alone; lane 2, λ -bacteriophage *Hind*III marker DNA; lane 3, pBR322 linearized with *Hind*III; lane 4, supercoiling mixture plus oxolinic acid (200 μ g/ml); lane 5, same as lane 4 but without oxolinic acid; lane 6, same as lane 4 but without proteinase K treatment; lane 7, same as lane 4 but with SDS omitted; lane 8, same as lane 4 but the reaction was stopped by heating at 80 $^{\circ}$ C followed by SDS and proteinase K treatment. Positions of the various forms of the plasmid DNA are indicated by bars: from the top downwards, relaxed, linear and supercoiled.

nalidixic acid at a fairly high concentration of 200 μ g/ml could only partially inhibit the supercoiling reaction. This concentration of nalidixic acid is approx. 100-fold higher than the minimum inhibitory concentration reported for *V. cholerae* 569B. We found that the minimum inhibitory concentration of nalidixic acid for the *V. cholerae* 569B used in this study was somewhat variable depending upon the medium and the conditions of growth of the cells. For comparison, it may be noted that *E. coli* gyrase is 50% inhibited by a concentration of 200 μ g of nalidixic acid/ml, which is 50-fold higher than the minimum inhibitory concentration reported for *E. coli* (Liu & Wang, 1978; Cozzarelli, 1980). Oxolinic acid, which like nalidixic acid also acts on the A subunit of *E. coli* DNA gyrase, was, however, found to be more potent and could completely inhibit the supercoiling reaction catalysed by the *V. cholerae* enzyme at a concentration of 10 μ g/ml (Fig. 2d).

In addition to its ability to inhibit *E. coli* DNA gyrase, oxolinic acid is known to be able to induce that enzyme to cleave double-stranded DNA if the supercoiling reaction is carried out in the presence of the drug and then a detergent such as SDS is added to the mixture (Sugino *et al.*, 1977; Gellert *et al.*, 1977). When intracellularly supercoiled pBR322 DNA was incubated with the DNA gyrase from *V. cholerae* under standard assay conditions for 60 min at 30 $^{\circ}$ C in the presence of 300 μ g of oxolinic acid/ml followed by the addition of EDTA, SDS and proteinase K to a final concentration of 10 mM, 2 mg/ml and 90 μ g/ml respectively, during a further incubation for 30 min at 37 $^{\circ}$ C no cleavage of double-stranded DNA could be observed (Fig. 3). Increasing the enzyme concentration 10-fold did not produce any change in the results. Control experiments run with an equivalent amount of *E. coli* DNA gyrase (Bethesda Research Laboratories) under identical conditions could induce strand cleavage in pBR322 DNA as has been reported previously (Sugino *et al.*, 1977; Gellert *et al.*, 1977) (results not shown).

Fig. 4. Inability of *V. cholerae* DNA gyrase to relax supercoiled pUC19 DNA

The gyrase reaction was carried out under standard assay conditions (see the text and legend to Fig. 2) except that ATP was omitted. Lanes 1 and 2, reaction carried out with *V. cholerae* DNA gyrase, 1.2 and 12 units respectively; lane 3, reaction carried out with 1 unit of *E. coli* DNA gyrase; lane 4, pUC19 DNA linearized with *EcoRI*; lane 5, supercoiled pUC19 DNA. Positions of the various forms of the plasmid DNA are indicated by bars: from the top downwards, nicked circular, relaxed, linear and supercoiled.

Apart from its supercoiling activity, gyrase from *E. coli* is known to be able to relax negatively supercoiled DNA if ATP is omitted from the reaction mixture and the Mg^{2+} concentration is raised to 6 mM or higher (Sugino *et al.*, 1977; Gellert *et al.*, 1977). Under identical conditions the DNA gyrase from *V. cholerae* failed to relax negatively supercoiled pUC19 DNA (Fig. 4). With the *E. coli* enzyme the relaxation reaction is inhibited by oxolinic acid but not by novobiocin (Gellert *et al.*, 1977), indicating that relaxation specifically requires the same subunit, namely subunit A of the enzyme, which is also involved in the breakage and rejoining of the double-stranded DNA. Since the *V. cholerae* enzyme appeared to be impaired in its ability to break and rejoin the DNA under the conditions in which the *E. coli* enzyme could do so, it is perhaps not very surprising that the *V. cholerae* enzyme was also found to be unable to carry out the relaxation reaction under the condition required by the *E. coli* gyrase to perform the same task.

In this paper we have described the purification and properties of DNA gyrase from *V. cholerae*, an enterobacterium belonging to the family Vibrionaceae. Several properties of this enzyme are reminiscent of its counterparts from other organisms examined so far. They all require ATP and Mg^{2+} for their action, they all are inhibited by anti-gyrase drugs and in the few cases examined

so far they are found to have multimeric structures of the type A_2B_2 (Klevan & Wang, 1980; Sugino *et al.*, 1980). What distinguishes the *V. cholerae* enzyme from its better-studied counterpart from *E. coli* is its inability to catalyse relaxation of the negatively supercoiled DNA or induce a cleavage of double-stranded DNA under the conditions in which the *E. coli* enzyme could do so. It is likely that *V. cholerae* gyrase is able to perform these reactions under a very different set of experimental conditions.

In conclusion, we have shown that, even though *E. coli* and *V. cholerae* diverged some 670 million years ago (Yamamoto *et al.*, 1987), the gyrases from these organisms are remarkably similar in nature, further strengthening the hypothesis that this enzyme is of fundamental importance for prokaryotes and has been conserved throughout evolution.

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