**Bacillus subtilis** DNA Gyrase: Purification of Subunits and Reconstitution of Supercoiling Activity

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DNA gyrase from *Bacillus subtilis* 168 was purified by affinity chromatography on novobiocin-Sepharose and shown to consist of two subunits, A and B, with molecular weights of 100,000 and 85,000, respectively. The B subunit, which contains novobiocin-sensitive ATPase activity, could complement the gyrA protein of *Escherichia coli*. No complementation was detected between the A subunit and the *E. coli* gyrB protein.

DNA gyrases are topoisomerases, which catalyze the supercoiling of relaxed closed circular DNA coupled to the hydrolysis of ATP (13). The enzyme from *Escherichia coli* (topoisomerase II) consists of two different subunits, A and B, encoded by the genes gyrA and gyrB, respectively (1, 2). Gyrase holoenzyme is considered to exist as a tetramer, $A_2B_2$, containing two each of the gyrA and gyrB gene products. The gyrA protein constitutes the target for nalidixic acid and oxolinic acid, whereas the gyrB protein is the target for novobiocin and related coumarin antibiotics. On the basis of inhibition studies with these drugs, the two components of DNA gyrase have been implicated in almost every transaction of bacterial DNA, and it is assumed that gyrase is present in all bacteria. However, structural studies have so far been carried out only with the enzyme from *E. coli* and *Micrococcus luteus* (4, 6). A supercoiling activity has also been detected in extracts of *Bacillus subtilis* (12), but only a partial purification of this enzyme has been achieved, and no structural characterization has been reported. This is apparently due to difficulties in obtaining sufficient quantities of highly purified gyrase by conventional procedures (9). We have recently described the isolation of novobiocin-binding proteins from *E. coli* by means of affinity chromatography on novobiocin-Sepharose (11). In this communication, we report the use of this method for the purification of the *B. subtilis* gyrase. The properties of the DNA gyrases from *B. subtilis* and *E. coli* will be compared.

*B. subtilis* 168 was grown in Luria broth and harvested at mid-log phase. Cells (40 g) were suspended in an equal volume of 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)–KOH (pH 8.0)–100 mM KCl and stored frozen at $-70^\circ$C. The frozen cell suspension was thawed and diluted with an equal volume of 25 mM HEPES–KOH (pH 8.0)–0.4 M sucrose–20 mM magnesium acetate–1 mM dithiothreitol–5 mM phenylmethylsulfonyl fluoride (PMSF). All operations were performed at 0 to 4°C. Lysozyme was added to a final concentration of 1 mg/ml, and the mixture was incubated for 2.5 h. One-third volume of 2 M KCl–1.5% Brij was added, and the incubation was continued for 15 min. The lysate was then centrifuged for 90 min at 30,000 rpm in a Beckman 45 Ti rotor. The supernatant was adjusted to a KCl concentration of 0.2 M by dilution with 25 mM HEPES–KOH (pH 8.0)–1 mM dithiothreitol–1 mM EDTA–0.5 mM PMSF–10% (wt/wt) ethylene glycol (buffer A) and applied to a novobiocin-Sepharose column (1.6 by 6 cm) equilibrated with buffer A (0.2 M KCl). The column was washed with starting buffer and eluted successively with buffer A containing 20 mM ATP–25 mM magnesium acetate–0.2 M KCl, buffer A (2 M KCl), and 5 M urea in buffer A (0.2 M KCl). The protein content of the eluate was determined (10), and protein-containing fractions were dialyzed against buffer A (0.05 M KCl).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that *B. subtilis* extracts contained two major novobiocin-binding proteins with molecular weights of 100,000 (100K protein) and 85,000 (85K protein), respectively (Fig. 1A and B). Both proteins were obtained in similar quantities, with a yield of 0.81 mg of 100K protein and 0.85 mg of 85K protein from 40 g of cells. The 100K protein was eluted from the novobiocin-Sepharose by high salt concentrations, whereas the 85K protein was bound more tightly and could only be de-
sorbed upon denaturation with 5 M urea. The binding of these two proteins to immobilized novobiocin was therefore strikingly similar to that of the E. coli gyrA and gyrB proteins, respectively (11). It should be noted, however, that the molecular weights of the novobiocin-binding proteins from B. subtilis were somewhat lower than the molecular weights of the subunits of the E. coli DNA gyrase (Fig. 1C). Furthermore, in contrast to E. coli extracts (11), no distinctive B. subtilis protein could be eluted from the affinity adsorbent with ATP, and no additional protein of lower molecular weight was detected in the urea eluate.

To determine whether the two novobiocin-binding proteins were actually components of B. subtilis DNA gyrase, we assayed their ability to catalyze the supercoiling of relaxed ColE1 DNA (Fig. 2). Neither protein by itself had any supercoiling activity, and the electrophoretic profiles shown in Fig. 2A and B are identical to that of an untreated control. However, in combination, both proteins carried out efficiently the conversion of relaxed closed circular DNA to supercoiled molecules comigrating with form I DNA (Fig. 2C). With a unit of gyrase defined as the activity that supercoils 1 μg of relaxed ColE1 DNA in 30 min at 30°C, the B. subtilis gyrase components had specific activities of 4 x 10^4 U/mg (100K protein) and 1 x 10^4 U/mg (85K protein), as estimated from serial dilutions of one component in the presence of an excess of the other.

The molecular weight and strength of binding to novobiocin-Sepharose suggested that the 100K protein corresponds to the gyrA protein and the 85K protein corresponds to the gyrB protein of E. coli. This was confirmed by showing that like the E. coli gyrB protein (11), the 85K protein of B. subtilis contained a DNA-independent ATPase activity ($K_m = 2.0$ mM) which was highly sensitive to novobiocin ($K_i = 10^{-7}$ M). The 100K protein stimulated the ATPase activity of the 85K protein in the presence of duplex DNA by lowering the apparent $K_m$ to 0.6 mM. These kinetic constants were nearly identical to those reported previously for the ATPase activity of the E. coli gyrB protein.

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of novobiocin-binding proteins from B. subtilis. Samples from the eluates of the novobiocin-Sepharose column were applied to polyacrylamide gels (10%) prepared by the method of Laemmli (5). The figure shows densitometer tracings of the Coomassie blue-stained gel: (A) 2 M salt eluate, (B) 5 M urea eluate, (C) E. coli DNA gyrase. Molecular weight reference markers were the subunits of RNA polymerase and DNA gyrase from E. coli, and bovine serum albumin.

FIG. 2. Supercoiling of relaxed ColE1 DNA. The assay for supercoiling was carried out as described previously (11). Reaction mixtures (25 μl) containing 2 μg of relaxed ColE1 DNA were incubated for 30 min at 30°C with 0.6 μg of 100K protein (A), 0.8 μg of 85K protein (B), and a combination of both (C). Samples were analyzed by agarose gel electrophoresis (3). The figure shows densitometer tracings of a photograph of an ethidium-stained gel. The arrows indicate the positions of supercoiled form I, open circular form II, and linear form III DNA, respectively.
(11). In accordance with the nomenclature of the *E. coli* enzyme, the 85K protein may therefore be designated as subunit B and the 100K protein may be designated as subunit A of *B. subtilis* gyrase.

To confirm this conclusion, we tested the supercoiling activity of intergeneric hybrids prepared from the isolated gyrase subunits of *B. subtilis* and *E. coli*. An active enzyme could be obtained by combining the *E. coli* A subunit with the *B. subtilis* B subunit (Fig. 3, lane 1). This interspecies complementation provided further evidence for the functional equivalence of the corresponding *B. subtilis* and *E. coli* subunits. However, the reciprocal combination of heterologous subunits did not result in an active holoenzyme. No supercoiling was detectable upon mixing the *B. subtilis* A subunit with the *E. coli* B subunit (Fig. 3, lane 2). Similarly, the *E. coli* gyrA protein had a DNA-dependent stimulatory effect on the ATPase activity of the *B. subtilis* B subunit, but the A subunit of *B. subtilis* did not stimulate the *E. coli* gyrB ATPase (data not shown). This could reflect the inability of the *B. subtilis* A subunit to interact with the nonhomologous B subunit. Apparently, gyrase activity requires specific interactions between the two different subunits of the holoenzyme and does not simply result from the cooperation of two separate proteins.

Although the B subunits of *B. subtilis* could complement the *E. coli* gyrA protein, it could not restore ColE1 DNA replication in an extract from the thermosensitive *E. coli* gyrB mutant LE316 (7, 8). However, addition of the *B. subtilis* B subunit in the presence of an excess of the *B. subtilis* A subunit resulted in a small but significant amount of plasmid DNA synthesis (Fig. 4). Agarose gel electrophoresis indicated that the average superhelical density of ColE1 DNA in an LE316 extract complemented with *B. subtilis* gyrase was slightly lower than that from an extract complemented with *E. coli* gyrB protein (data not shown). These findings support the notion that the capacity of plasmid DNA to carry out rounds of replication is strongly affected by its superhelical state, which results from the balance of supercoiling and relaxing topoisomerase activities present in crude extracts. Apparently, the *E. coli* gyrase is more efficient in counteracting the homologous relaxing enzyme (*E. coli* topoisomerase I) than either the *B. subtilis* gyrase or the chimeric enzyme.

In conclusion, we have shown that affinity chromatography on novobiocin-Sepharose can be used to purify DNA gyrase from the taxonomically widely separated bacteria *E. coli* and *B. subtilis*. The *B. subtilis* enzyme was found to differ from the *E. coli* and *M. luteus* gyrases by the smaller size of its subunits. Otherwise, the structural and catalytic properties of these topoisomerases appeared to be very similar. Intergeneric complementation studies revealed, however, specific interactions between homologous gyrase subunits. Furthermore, these studies, taken together with previous results (7), suggest that the gyrase activity of a bacterial cell has to be precisely attuned to the activity of the relax-
ing enzyme(s) to maintain the native superhelical state of its DNA.

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