Purification and Characterization of DNA Topoisomerase IV in *Escherichia coli*

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The subunits of topoisomerase IV (topo IV), the ParC and ParE proteins in *Escherichia coli*, were purified to near homogeneity from the respective overproducers. They revealed type II topoisomerase activity only when they were combined with each other. In the presence of Mg2+ and ATP, topo IV was capable of relaxing a negatively or positively supercoiled plasmid DNA or converting the knotted P4 phage DNA, whether nicked or ligated, to a simple ring. However, supercoiling activity was not detected. The topoisomerase activity was not detectable when the purified ParC and ParE proteins were combined with the purified GyrB and GyrA proteins, respectively. This is consistent with the result that neither a parC nor a parE mutation was compensated by transformation with a plasmid carrying either the gyrA or the gyrB gene. Simultaneous introduction of both the gyrA and gyrB plasmids corrected the phenotypic defect of parC and parE mutants. The results suggest that DNA gyrase can substitute for topo IV at least in some part of the function for chromosome partitioning. Antisera were prepared against the purified ParC, ParE, GyrA, and GyrB proteins and used to investigate cellular localization of these gene products. ParC protein was found to be specifically associated with inner membranes only in the presence of DNA. This result suggests that one of the functions of topo IV might be to anchor chromosomes on membranes as previously proposed for eukaryotic topoisomerase II.

The mechanism of chromosome partitioning in *Escherichia coli* has been one of the most intriguing themes in the study of the bacterial cell cycle and cell division. In *E. coli*, the process of cell division proceeds apparently in the absence of the bacterial cell cycle and cell division. In *E. coli*, the process of cell division proceeds apparently in the absence of chromosome replication with cell division probably involving chromosome-membrane interactions. Involvement of the cell surface structure in chromosome segregation was proposed by Jacob and his colleagues(1963) in their "replicon model." Since then, the attachment of chromosomes to the cell surface has been believed to be the mechanism for chromosome partitioning in prokaryotic cells but without a conclusive experimental result.

Investigations into the mechanism of chromosome partitioning have been undertaken by utilizing thermosensitive par mutants of *E. coli*. The par mutants are characterized by formation of large aggregated nucleoids at the restrictive temperature, and five classes of par mutations have been identified: parA, parB, parC, parD, and parE (Hirata et al., 1968, 1971; Hussain et al., 1987; Kato et al., 1988, 1990). It has been found that the Par phenotype described as parA and parD is due to mutations in the gyrB and gyrA genes, respectively (Kato et al., 1989; Hussain et al., 1987), and parD is most probably an allele of dnaG (for DNA primase) (Norris et al., 1986). On the other hand, parC and parE, both located around 65 min on the *E. coli* genetic map, represent new genes essential for chromosome partition. The gene products of parC and parE have considerable homology to the A and B subunits of DNA gyrase in primary structure. When crude cell lysates were prepared from ParC and ParE overproducers and combined, they showed enhanced topoisomerase activity, and the properties of ParC and ParE have suggested that ParC and ParE are components of a new topoisomerase, designated topo IV, which possibly belongs to the class of type II topoisomerases (Kato et al., 1990).

DNA gyrase, a type II topoisomerase in *E. coli*, has been shown to take part in chromosome partitioning (Steck and Driica, 1964). It is conceivable that circular replicons such as *E. coli* chromosomes might remain linked topologically in catenanes as observed in small plasmids (Sakakibara et al., 1976). Even eukaryotic cells with linear replicons require topoisomerases for resolving intertwined replicons. Mutants defective in a type II topoisomerase of *Sacharomyces cerevisiae* and *Schizosaccharomyces pombe* show abnormalities in chromosome segregation; catenated plasmids as well as unsegregated chromosomes have been observed in an *S. cerevisiae* topo II mutant, and topoisomerases can resolve the intertwined replicons *in vitro* (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1984, 1986). The major function of the topoisomerase in chromosome segregation may be topological resolution of intertwined replicons. Recent researches, however, have illuminated other functions of topoisomerases (Wang, 1991; Wang et al., 1990). Topoisomerases may act as anchors in a nuclear scaffold (Gasser et al., 1986; Adachi et al., 1989; Spery et al., 1989) and contribute to chromosome condensation (Uemura et al., 1987; Adachi et al., 1991). The lack of a topoisomerase function enhances some kinds of recombination in yeast, suggesting that topoisomerases contribute to the maintenance of genome stability (Christman et al., 1988; Kim and Wang, 1989; Wallis et al., 1992).

The abbreviations used are: topo, topoisomerase; m-AMSA and o-AMSA, 4'-9-acridinylamino)methanesulfon-m-anisidine and o-anisidine, respectively; BSA, bovine serum albumin.

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1989; Aguiler and Klein, 1990). The function of a topoisomerase in prokaryotes has been suggested in the interaction of DNA gyrase with the par region of plasmid pSC101, and at this par region, the plasmid is associated with a cell membrane (Gustafsson et al., 1983; Wahle and Kornberg, 1988). The par region is required for stable partition (topographical segregation) of replicated plasmids into daughter cells but not for decatenation (topological resolution). DNA gyrase may function to maintain superhelicity of replications, since plasmids lacking the par region are more relaxed and unstable (Miller et al., 1990).

In order to have an insight into the functions of top IV, we examined the topoisomerase activity and cellular localization of this protein. Although top IV is homologous to DNA gyrase in amino acid sequence, top IV showed a relaxation activity but not the supercoiling activity of DNA gyrase.

Subunits were not interchangeable between DNA gyrase and top IV, either in vivo or in vitro. Nevertheless, an increase in gene dose of both gyrA and gyrB compensated the phylogenetic defect of parC and parE, suggesting that DNA gyrase can associate with the inner membrane. The ParC protein revealed a unique property of DNA-dependent association with inner membranes. Prokaryotic type II topoisomerases, top IV and parE, may be involved in anchoring chromosomal DNA and forming chromosome loops as suggested in regard to eukaryotic topoisomerase II.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, and Culture Media**—The E. coli strains used were: C600 (F-, thi thr leu B lac Y ona supE43), DH1 (F-, recA1, endA1, gyrA96 thi-1 hsdR17 supE44) (Sambrook et al., 1989), EJ812 (parC1215, a derivative of C600) (Kato et al., 1990), W3110parE10 (Kato et al., 1990), YN2942 (TAP106 (A inc-clII) BAM: N::kan cl857 (cro-bla1)) (Inda et al., 1989), and SW1053 (Mizuchi et al., 1984).

Plasmids used were pSY334 (Yasuda and Takagi, 1983), pJL6 (Inda et al., 1989), pJB11 (Yamagishi et al., 1986), pMK47, pMK90 (Mizuchi et al., 1984), pACYC184 (Chang and Cohen, 1978), pUC4K (Vieira and Messing, 1982), pK625, pK2000, and pK2020 (Kato et al., 1990).

Bacteria were grown routinely in LB broth and antibiotic medium E. coli top IV and plasmid DNA, manipulation of DNA, and transformation were as described (Sambrook et al., 1989) except that cells from a 1.6-liter culture in a suspension of 28 ml of 10 mM Tris-HCl, pH 8.0, 5 mM potassium chloride, 10% sucrose were added to NaCl 10 mM, 0.15 M NaCl, and 10% glycerol, and loaded onto a 2-

**Preparation of GyrA and GyrB Proteins**—DNA gyrase A and B proteins purified with DEAE-Sepharose were loaded on a hydroxyapatite column (Tonen Corp., Tokyo, Japan) (3.46 cm × 10 cm) equilibrated with KP2 buffer (50 mM potassium phosphate, pH 6.8, 1 mM 2-mercaptoethanol, 0.3 mM NaCl, 10% glycerol), and proteins were eluted with a 200-ml gradient of potassium phosphate (50-100 mM) in KP2 buffer. The fractions containing GyrA protein were pooled, concentrated with a Q-Sepharose fast flow column (Pharmacia LKB Biotechnology Inc.) (0.5 cm × 5 cm) equilibrated with TE buffer containing 0.3 mM NaCl, and protein was eluted with 0.15 M NaCl. After addition of 0.1 volume of 75% glycerol, the concentrated fraction was loaded onto a Sephacryl S400 column (Pharmacia) (2.4 cm × 85 cm) and dialyzed against the same buffer. The ParC fractions were pooled, concentrated using the Q-Sepharose fast flow column as above, and frozen quickly in small aliquots with liquid nitrogen.

The crude lysate of the ParC overproducng strain was also prepared as described (Kato et al., 1990) except that cells from a 1.6-liter culture in a suspension of 28 ml of 10 mM Tris-HCl, pH 8.0, 5 mM potassium chloride, 10% sucrose were added to NaCl 10 mM, 0.15 M NaCl, and 10% glycerol, and loaded onto a 2-

**Preparation and Manipulation of DNA**—The techniques for preparation of plasmid DNA, manipulation of DNA, and transformation were as described (Sambrook et al., 1989).

**Purification of ParC and ParE Proteins**—All operations were performed at 4 °C. The ParC and ParE proteins were purified by detecting protein bands in SDS electrophoretic gels.

The crude lysate of the ParC overproducing strain was prepared from an 800-ml culture as described (Kato et al., 1990) except that cells from a 1.6-liter culture in a suspension of 28 ml of 10 mM Tris-HCl, pH 8.0, 5 mM potassium chloride, 10% sucrose were added to NaCl 10 mM, 0.15 M NaCl, and 10% glycerol, and loaded onto a 2-

**Preparation of GyrA and GyrB Proteins**—DNA gyrase A and B proteins were purified with DEAE-Sepharose and loaded on a hydroxyapatite column (Tonen Corp., Tokyo, Japan) (3.46 cm × 10 cm) equilibrated with KP4 buffer (25 mM potassium phosphate, pH 6.8, 1 mM 2-mercaptoethanol, 10% glycerol) instead of a valine-Sepharose column. The hydroxyapatite column was washed with 10 column volumes of KP4 buffer, and proteins were eluted with a KP5 buffer (200 mM potassium phosphate, pH 6.8, 1 mM 2-mercaptoethanol, 10% glycerol). The fractions containing GyrA protein were pooled and concentrated with a DEAE-Sepharose column (Pharmacia) (0.5 cm × 2.5 cm). The pooled fraction (2 ml) was miliated with addition of 4 ml of 2 M Tris-HCl, pH 8.0, 0.5 ml of 0.5 M EDTA, and 10% glycerol and loaded onto a 2-

**Purification of GyrA and GyrB Proteins**—DNA gyrase A and B proteins were purified from overproducing strains as described (Mizuchi et al., 1984) with several modifications as follows.

After purification of GyrB protein, the last step of leucine-agarose chromatography was omitted, and the GyrB fraction (15 ml) eluted from a hydroxyapatite column was diluted with addition of 4.5 ml of 2
RESULTS

Purification of ParC and ParE Proteins—Overproducers for ParC and ParE proteins have been constructed, and the enhanced relaxation activity of their lysates in vitro has suggested the overproduction of the active proteins (Kato et al., 1990). The ParC and ParE proteins were separately purified to near homogeneity from each overproducer by detecting the ParC and ParE protein bands in SDS electrophoretic gels (Fig. 1, Table 1). Topoisomerase activity was detected only in the presence of both proteins (Fig. 2A). At the final purification stage, the ParC and ParE fractions eluted from the chromatographic column were assayed for the topoisomerase activity in the presence of the other purified subunit, and the amount of the ParC and ParE protein was shown to correlate with the degree of relaxation activity (data not shown). These results also confirm that topo IV consists of the ParC and ParE proteins. When the overproducing strains were lysed with a French press or sonication, ParC protein was stable, but ParE protein was degraded, especially in the presence of Mg²⁺ (data not shown). The instability of the ParE protein depends on the method of lysis. ParE protein was more stable when cells were lysed by freezing and thawing (Fig. 1B, lane 1). The protease which might degrade the ParE protein has not been identified. OmpT protease is not responsible because the instability was little influenced by an ompT⁺ mutation (data not shown). Unless the ParC and ParE proteins were purified in the presence of NaCl at a concentration higher than 0.15 M, they aggregated. Even in the buffer containing 0.3 M NaCl, these proteins may not exist as monomers, since they were eluted near the void volume in gel filtration with

![Fig. 1. Purification of ParC and ParE proteins](image-url)

Proteins at each purification step (see Table I and "Experimental Procedures") were examined by SDS polyacrylamide (12%) gel electrophoresis. A, purification of ParC protein. Lane 1, cell extract (step 1); lane 2, cell extract after Polyni P treatment; lane 3, dialyze after ammonium sulfate precipitation; lane 4, proteins extracted from the precipitate of the dialysate (step 2); lane 5, ParC fraction of hydroxylapatite chromatography (step 3); lane 6, ParC fraction of Sephacyr S400 chromatography (step 5); lane 8, molecular mass markers (phospholipase B, 97.4 kDa; BSA, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa). B, purification of ParE protein. Lane 1, cell extract prepared by freezing and thawing (step 1); lane 2, cell extract treated with Polyni P; lane 3, dialyze after ammonium sulfate precipitation; lane 4, proteins extracted from the precipitate of the dialysate (step 2); lane 5, ParE fraction of hydroxylapatite chromatography (step 3); lane 6, ParE fraction of Q-Sepharose fast flow chromatography (step 4); lane 7, ParE fraction of Sephacyr S200 chromatography (step 5); lane 8, molecular mass markers as in A.
Sephacryl S400 and S200 (exclusion limit of 8,000 and 250 kDa, respectively). Even though the purified ParC and ParE proteins may be aggregated or polymerized, they can function as a topoisomerase as described in the next section.

**Topoisomerase Activity of Topo IV**—As described in the previous work, the combined crude cell lysates of the ParC and ParE overproducers showed an enhancement in relaxation activity (Kato et al., 1990). Using the purified ParC and ParE proteins, we were able to detect the relaxation activity when both proteins were mixed in the presence of ATP and MgCl₂ (Fig. 2A), but supercoiling activity was not detected (data not shown). The optimal NaCl concentration for the relaxation activity was 70–80 mM (Fig. 2B). MnCl₂, although less effective, was able to substitute for MgCl₂ (Fig. 2A). As shown in Fig. 2A, especially in the absence of ATP, accumulation of possible cleavable complexes was observed even in the absence of inhibitors. The accumulation of possible cleavable complexes was also observed when lower amounts of ParC or ParE proteins were present in the reaction mixture (Fig. 2C). The result of a titration experiment with ParC and ParE proteins suggests that the molar ratio of the ParC to the ParE protein in topo IV might be 1:1 (Fig. 2C).

In order to know whether topo IV can relax positively supercoiled DNA, topo IV was incubated with the positively supercoiled DNA prepared with reverse gyrase, and the product was analyzed by two-dimensional gel electrophoresis. Positively supercoiled DNA can be clearly distinguished from negatively supercoiled DNA by two-dimensional gel electrophoresis; in the second dimension, positively supercoiled DNA has a higher mobility due to the increase in the superhelical density by ethidium bromide, while the migration of negatively supercoiled DNA was retarded because of relaxation by ethidium bromide. In the presence of a rather low concentration of ethidium bromide, since highly negative supercoils run as fast as highly positive supercoils (Fig. 2D, (ii), lanes 1 and 4), only moderately negative supercoils can be distinguished from positive supercoils. The result clearly showed that topo IV could also relax positively supercoiled DNA (Fig. 2D).

In *E. coli*, topo IV is the first example of an enzyme that can relax positively supercoiled DNA, since bacterial topo I and intact DNA gyrase cannot (Gellert, 1981; Wang, 1985). DNA gyrase has no activity for relaxing positively supercoiled DNA in the absence of ATP or βγ-imido-ATP but can convert positively supercoiled DNA to negatively supercoiled DNA by supercoiling activity in the presence of ATP (Gellert, 1981).

Unknotting activity was investigated using knotted P4 phage DNA as substrate. DNA extracted from a P4 mutant phage is knotted and nicked in both strands, and both type I and II topoisomerases can convert it to an unknotted circle, although P4 phage DNA ligated *in vitro* can be unknotted only by type II topoisomerases. As shown in Fig. 2E, topo IV can untangle the knotted DNA, either nicked or ligated; in this figure the knotted P4 DNA appears as a smear, while the unknotted phage DNA is detected as a sharp band in agarose gel electrophoresis. To confirm the structure of the substrate DNA, the nicked and the ligated DNA were incubated at 75°C for 5 min. After the heat treatment, the ligated phage DNA was not changed in electrophoretic appearance, while the nicked DNA was converted to a simple ring to form a single distinct band. The unknotting activity, as well as the relaxation activity, of topo IV required both subunits, ATP and MgCl₂ (data not shown).

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**Fig. 2F shows the effects of inhibitors. The inhibitors of DNA gyrase, oxolinic acid and novobiocin, inhibited the relaxation activity of topo IV at almost the same concentration as for DNA gyrase. (The detailed analysis of inhibition of topo IV activity by inhibitors will be described elsewhere.) This result is consistent with the fact that the homology in amino acid sequence is higher between topo IV and DNA gyrase than between topo IV and eukaryotic type II topoisomerases (Kato et al., 1990).**

Compensation of the parC and parE Mutations by Increasing Gene Dosage of Both gyrA and gyrB—The amino acid sequence homology between topo IV and DNA gyrase suggested the possibility that the subunits of topo IV might be substituted by the subunits of DNA gyrase. To test this possibility, topoisomerase activities were investigated *in vitro* for relaxation, supercoiling, and unknotting in combinations of ParC and GyrB or ParE and GyrA. No activity was detected, at least under the reaction conditions for DNA gyrase or topo IV (data not shown), suggesting that the subunits of topo IV and DNA gyrase are not interchangeable.

The results *in vitro* are consistent with those obtained in

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**TABLE I**

| Purification step | Volume | Total protein | Yield | Purification* |
|------------------|--------|---------------|-------|--------------|
| ParC             |        |               |       |              |
| 1. Extract       | 30     | 244           | 100   | 1.0          |
| 2. Polyamin-ammonium sulfate | 1.9 | 36.9           | 63.4  | 4.0          |
| 3. Hydroxylapitate | 39.2  | 6.7           | 54.9  | 19.3         |
| 4. Q-Sepharose fast flow | 34.3  | 2.1           | 20.7  | 22.9         |
| 5. Sephacryl S400 | 6.3    | 0.4           | 12.2  | 71.4         |
| ParE             |        |               |       |              |
| 1. Extract       | 22     | 250           | 100   | 1.0          |
| 2. Polyamin-ammonium sulfate | 1.9 | 19.8           | 28.7  | 3.7          |
| 3. Hydroxylapitate | 49.0  | 7.4           | 24.5  | 8.3          |
| 4. Q-Sepharose fast flow | 29.4  | 1.4           | 12.5  | 22.9         |
| 5. Sephacryl S200 | 4.4    | 0.12          | 1.89  | 41.7         |

* Amounts of ParC and ParE proteins were estimated by quantifying and comparing the intensity of the ParC and ParE bands on Western blot membranes with that of the purified ParC and ParE proteins as standards. The total amount of protein in the cell extracts was taken as 100%. One unit of topoisomerase activity was defined as the amount of enzyme required to fully relax 0.55 µg of supercoiled pBR322 plasmid DNA under standard assay conditions. The specific activities of the final samples were 1 × 10⁴ units/mg for each of the ParC and ParE proteins.
2-7, 25680 protein plus 200, 100, 50, 25, 12.5, 6.25, or 3.13 ng of the ParE protein was added to the reaction mixture, and incubation was continued for 15 min at 37 °C.  

Supercoiled DNA were used as substrate for the relaxation assay of ParC and ParE subunits, ATP, and the titration of NaCl was changed.

The concentration of NaCl was varied to study the effect of NaCl concentration. ParC and ParE proteins were contained in the reaction mixtures. The concentration of NaCl (25 mM (lane 2), 45 mM (lane 3), 65 mM (lane 4), 85 mM (lane 5), and 105 mM (lane 6)). C, titration of the ParC and ParE proteins in the relaxation assay. Removal of negative supercoils was measured in a standard mixture as described under "Experimental Procedures." Reaction mixtures in (i) contained no enzyme (lane 1) or 200 ng of ParE plus 200, 100, 50, 25, 12.5, or 6.25 ng of ParC (lanes 2–7, respectively); reactions in (ii) contained 200 ng of the ParC protein plus 200, 100, 50, 25, 12.5, 6.25, or 3.13 ng of the ParE protein (lanes 1–7, respectively). D, relaxation of negatively and positively supercoiled pBR322 DNA. Negatively (ii), lanes 2–4 and (ii), lanes 1–3) and positively (i), lanes 5 and 6 and (ii), lanes 4 and 5) supercoiled DNA were used as substrate for the relaxation assay of topo IV. (200 ng each of the ParC and ParE proteins were contained in the reaction mixtures.) Positively supercoiled DNA was prepared with reverse gyrase (Nakasu and Kikuchi, 1985). The same samples were examined by one-dimensional gel electrophoresis, the samples were electrophoresed first in the vertical direction (from top to bottom) in the standard condition and then reelectrophoresed in the horizontal direction (from left to right) in the same buffer containing 0.02 μg/ml ethidium bromide. (i), lane 1, λ phage DNA digested with HindIII; (ii), lanes 2 and 5, and (ii), lanes 1 and 4, no enzymes. DNA was incubated with topo IV (i), at 30 °C for 30 min (lane 3 and (ii), lane 2), at 37 °C for 60 min (i) lane 4 and (ii) lane 3, at 30 °C for 60 min (i) lane 6 and (ii) lane 5). All samples were treated with 1 ml proteinase K after incubation at 30 °C for 10 min. E, unknotting of knotted P4 phage DNA. The knotted and nicked P4 DNA (lanes 1–4) and the nicked P4 DNA that was ligated in vitro (lanes 5–8) were used for the unknotting assay. (100 ng each of the ParC and ParE proteins were contained in the reaction mixtures.) Lanes 1 and 5, no enzyme; lanes 2 and 6, incubated with no enzyme at 75 °C for 5 min; lanes 3 and 7, incubated with top0 IV at 37 °C for 10 min; lanes 4 and 8, digested with EcoRI; lane 9, λ-phage DNA digested with HindIII. The nicked and knotted DNA was converted to simple nicked DNA by treatment with top0 IV, as indicated. The results shown were representative of at least three experiments. F, effect of inhibitors on topo IV-catalyzed relaxation of negatively supercoiled pBR322. Positively supercoiled pBR322 was incubated with topo IV (200 ng each of the ParC and ParE proteins) in the presence of inhibitors. (i), lane 1, no enzyme. DNA was incubated with topo IV (lanes 2) in the absence of inhibitors or presence of novobiocin (lane 3, 5 μM; lane 4, 50 μM; lanes 5 and 6, 500 μM), ethoxylated (lanes 7, 50 μM; lanes 8 and 9, 500 μM) or ethylmaleimide (lanes 10), 0.5 μM; lane 11, 5 μM). Lanes 6 and 9, samples were incubated with proteinase K at 30 °C for 10 min after incubation with topo IV. Lane 12, λ phage DNA digested with HindIII. (ii), lane 1, no enzyme. pBR322 was incubated with topo IV (lanes 2, 7, and 12) in the absence of inhibitors or presence of m-AMS (lane 3, 10 μg/ml; lane 4, 10 μg/ml; lane 5, 1 μg/ml; lane 6, 0.1 μg/ml; lane 7, 100 μg/ml; lane 8, 10 μg/ml; lane 9, 10 μg/ml; lane 10, 1 μg/ml; lane 11, 0.1 μg/ml; and VP16 (lane 13, 100 μg/ml; lane 14, 10 μg/ml; lane 15, 1 μg/ml; lane 16, 0.1 μg/ml).
ParC, GyrA, and GyrB proteins. When the amounts of the subunits were estimated by quantifying the intensity of the bands on Western blot membranes, in the presence of EDTA, almost all of the subunits of topo IV and DNA gyrase were detected in the soluble fractions; in contrast, in the presence of Mg²⁺, about half of both GyrA and GyrB and almost all of ParC were localized to the membrane fraction. As described in the previous section, ParE protein was degraded in the presence of Mg²⁺ so that it was difficult to study its cellular localization, although the localization of the degraded protein appeared almost the same as that of ParC protein (Fig. 3).

In order to know whether the ParC protein in the crude membrane fraction was really associated with the membrane or not, the crude membrane was further fractionated. Because in the presence of Mg²⁺ the crude membrane fraction contains a lot of ribosomes, we adopted the flotation gradient method to fractionate the crude membrane (Ishidate et al., 1986). In the flotation gradient method, a crude membrane fraction is placed near the bottom under the sucrose gradient set up stepwise and centrifuged as described under “Experimental Procedures.” By this procedure, only membrane fractions were floated, leaving ribosomal fractions at the starting position; as a result, the inner and outer membrane fractions were separated and detected without interference by the ribosomal fraction. The crude membrane fraction subjected to the flotation gradient was prepared by sedimenting through 25% sucrose cushioned with 60% sucrose on which crude membranes were sedimented in a layer. As described in Fig. 4, the crude membranes were fractionated into two major peaks of membranes by flotation through the sucrose gradient. Distribution of the activity of a marker enzyme for the inner membrane, D-lactate dehydrogenase, and the electrophoretic profile of the outer membrane proteins suggested that the upper peak contained mainly inner membranes and that a major portion of the outer membrane was found in the lower peak. Using the antiserum against ParC protein, we studied the distribution of the ParC protein. To our surprise, very different results were obtained when fractionation was carried out in the presence or absence of DNase I. The majority of ParC protein remained at the starting position, but not in the floated fractions, in the presence of DNase I (Fig. 4A), while the ParC protein was detected in the inner membrane fraction in the absence of DNase I (Fig. 4B). This result suggests that the ParC protein is associated with the inner membrane only in the absence of DNase I, i.e. in the presence of DNA. The distribution of the GyrA and GyrB proteins, about half of which were localized to the crude membrane fraction in the presence of Mg²⁺ (see above), was not affected by DNase, i.e. similar amounts of these proteins were found in the inner membrane fraction regardless of DNase I (data not shown).

The result suggests that most of the gyrase subunits found in the crude membrane fraction, which account for about half of the total cellular subunits, are associated with the inner membrane. The existence of ParC in the inner membrane fraction only in the absence of DNase I suggests that the association of ParC with the inner membrane requires the participation of DNA. To confirm the role of DNA in the interaction between ParC protein and the inner membrane, a further analysis was carried out. The fractionated inner membrane fraction containing ParC protein was divided into two portions, and DNase I was added to one of them. The two portions were then subjected to a second flotation gradient fractionation. As shown in Fig. 5, with the DNase I-treated sample, about half of the ParC was left at the starting position, while almost all of the ParC of the other sample, to which DNase I was not added, was again recovered in the inner membrane fraction. Vesicle formation of the inner membrane may be the reason why only half of the ParC protein was dissociated from the inner membrane. These results suggest that DNA is contained in the ParC inner membrane fractions and that DNA is necessary for the association of ParC with the inner membrane.

**DISCUSSION**

In *E. coli*, two genes, *parC* and *parE*, have been found to code for a new type II topoisomerase, topo IV. Their amino acid sequences are homologous to those of the GyrA and GyrB subunits of DNA gyrase, respectively, and an enhancement in relaxation activity was detected when crude cell lysates prepared from their overproducers were mixed (Kato et al., 1990). Therefore, there are two type II topoisomerases in *E. coli*, and both of these enzymes are essential for chromosome segregation, because the topo IV mutants showed the Par phenotype at the nonpermissive temperature as observed in some DNA gyrase mutants. Purification and characterization of topo IV were necessary to uncover the function of topo IV and to answer the question why more than one type II topoisomerase is needed for chromosome segregation.

The subunits of topo IV, ParC and ParE proteins, were purified from the overproducers for these subunits, and the purified proteins were shown to have topoisomerase activity. The coincidence of distribution of the topoisomerase activity with that of ParC or ParE protein in chromatographic fractionation confirmed that ParC and ParE proteins constitute topo IV and that these subunits are the minimum components required to form a complex having topoisomerase activity. Both ParC and ParE proteins were found to become insoluble under low ionic strength conditions, and the purified proteins may aggregate or polymerize, even in the presence of a rather high concentration of NaCl, as suggested by the elution pattern on gel filtration. This feature might be an artifact due to overproduction, or it may be inherent in the nature of these proteins. In the presence of EDTA, most of the ParC protein of a cell that overproduced neither of them was found to be in a soluble fraction. However, when the soluble fraction was further fractionated by centrifugation through a sucrose gradient, ParC was detected at a slightly lower position than...
FIG. 4. Interaction of ParC with inner membranes. The crude membrane fraction of E. coli DH1 was further fractionated into the inner and outer membrane as described under "Experimental Procedures." A, fractionation in the presence of DNase I. DNase I was added before disruption with a French press (see "Experimental Procedures"). After centrifugation, samples were collected from top to bottom of the tube (corresponding to left to right in the panels). (i), absorbance at 280 nm; (ii), the activity of D-lactate dehydrogenase (a marker of inner membrane); (iii), protein profiles by SDS gel electrophoresis; (iv), the ParC proteins were detected using an anti-ParC serum by Western blot.

B, fractionation in the absence of DNase I. Fractionation, sampling, measurement of absorbance at 280 nm (i), and D-lactate dehydrogenase activity (ii), analysis of the protein profiles (iii), and detection of the ParC protein (iv) were carried out as in A except that DNase I was not added during fractionation.

FIG. 5. Dissociation of the ParC protein from inner membranes by treatment with DNase I after fractionation. The inner membrane containing ParC proteins was prepared by flotation gradient fractionation in the absence of DNase I. DNase I was added to half of the isolated inner membrane (A) but not to the other half (B) (see "Experimental Procedures"). Samples were again fractionated through a flotation gradient. Absorbance at 280 nm (i) was measured, and the ParC proteins were detected by Western blot (ii).

Most of the other soluble proteins (data not shown). DNA gyrase is thought to function as a tetramer containing two GyrA and two GyrB subunits, and further experiments would be necessary to clarify the composition of topo IV 
vivo.

ParE protein was found to be degraded when cells were drastically disrupted by French press or sonication. The degradation might be due to a protease, because sonication of the purified ParE proteins did not cause degradation 
in vitro (data not shown). In E. coli, an enzyme named topo II', composed of GyrA and a small protein fragment derived from GyrB, has been purified. Both GyrB and ParE might have some common structures susceptible to a preferential attack by proteases. The physiological role of the instability, if any, remains unknown.

Purified topo IV was found to have relaxation activity but not supercoiling activity 
in vitro, though the amino acid sequence was homologous to that of DNA gyrase. Topo IV as well as topo I might function in relaxation 
in vivo, in contrast to the action of DNA gyrase. A defect of topo I was compensated by increasing the gene dose of both parC and parE (Kato et al., 1990). One of the suppressor mutations of topA (topo I), toc, which was reported to be associated with ampli-
Inhibition of DNA gyrase might lead to cell death. Much higher concentration of the inhibitors might be needed for the lethal inhibition of top IV than for that of DNA gyrase. A more precise analysis of the effects of inhibitors is necessary and is in progress.

Immunological studies of the localization of top IV and DNA gyrase uncovered the interesting feature of interaction with the inner membrane. The ParC protein, especially, was suggested to interact with the membrane only in the presence of DNA. The ParC-inner membrane interaction may not be a nonspecific interaction, in which the ParC protein merely binds to nonspecific DNA and the DNA is associated with the membrane, because major DNA fraction was recovered from the soluble fraction but not from the membrane fraction after disruption of cells with a French press at rather high pressure used in this experiment. The ParC protein might anchor chromosomes to the inner membrane as eukaryotic top II is thought to anchor chromosomes to nuclear scaffolds. Since a mutant defective in the ParC-DNA-inner membrane interaction has not been isolated, the function involved in the interaction has not yet been clarified as is the case with eukaryotic top II. Among the parC mutants isolated so far, there may be mutants defective in the ParC-DNA-inner membrane interaction; we are now characterizing the mutants. In eukaryotes, chromosomes attach to the nuclear scaffold via specific DNA regions named SAR or MAR; by analogy, the ParC-inner membrane interaction might need specific DNA regions. The amount of ParC protein in a cell was estimated using antiserum and was almost the same as those of GyrA and GyrB according to the results of Gellert (1981) (about 500–1000 molecules/cell for each subunit; the result was referred to by Yang and Ames (1988)). There may be a lot of possible specific sites like SAR. One of the candidates is the family of REP sequences, which were reported to be specific binding sites of DNA gyrase (Yang and Ames, 1988). However, the specific interaction between DNA gyrase and repetitive extragenic palindromic sequences was not confirmed by the results of other groups (Higgins et al., 1988; Gilson et al., 1990). Other specific sequences or specific structures in chromosomes may be responsible for binding of top IV and DNA gyrase. Identification of the DNA regions and membrane component(s) that are necessary for interaction between type II topoisomerases, DNA gyrase and top IV, and the inner membrane is essential for understanding the function of type II topoisomerases in vivo.

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