Mutational Analysis of *Escherichia coli* Topoisomerase IV

I. SELECTION OF DOMINANT-NEGATIVE *parE* ALLELES*

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In order to define regions of ParE, one of the two subunits of topoisomerase IV, that are involved in catalysis during topoisomerization, we developed a selection procedure to isolate dominant-negative *parE* alleles. Both wild-type *parC* and mutagenized *parE* were expressed from a tightly-regulated *lac* promoter on a moderate-copy plasmid. Mutated *parE* alleles were rescued from those plasmids that caused IPTG-dependent cell death. The mutant ParE proteins could be divided into two groups when reconstituted with ParC to form topoisomerase IV, those that elicited hyper-DNA cleavage and those that affected covalent complex formation.

Type II topoisomerases utilize the cycle of ATP binding, hydrolysis of the β-γ phosphodiester bond, and release of ADP and P1 to drive a series of conformational changes that allow the enzyme to pass one DNA helix through a transient protein-bridged, double-strand break in either another segment of the same DNA helix or a different DNA helix. This results in an alteration of the linking number of the DNA. In general, if the same DNA ring contains both the segment of DNA where the break is made and the segment that is passed through the break, the net result is the removal of supercoils. If these two segments of DNA are on different molecules, catenation or decatenation results. These properties make topoisomerases required for essentially all macromolecular processes that operate on DNA in the cell (1–3).

The basic sequence of events necessary for one round of topoisomerization has been outlined and incorporated into the two-gate model (4). Capture of a segment of DNA (the T segment) to be transported through the DNA break (the DNA gate) is accomplished by ATP binding-dependent dimerization of two halves of the enzyme (the N gate). This initiates a concerted series of events where the T segment is then forced through the DNA gate, which then closes, resulting in the passage of the T segment to the interior of the enzyme. Release of the T segment from the enzyme occurs upon opening of the C gate. ATP hydrolysis results in re-opening of the N gate, thereby resetting the enzyme for another cycle.

The prokaryotic and eukaryotic enzymes share extensive amino acid sequence similarity and are organized in a similar fashion (5, 6). The eukaryotic enzymes are homodimers of a single polypeptide chain that contain an N-terminal ATP-binding domain and a C-terminal DNA cleavage domain. In the prokaryotic enzymes, these domains are on separate subunits and the protein is a heterotetramer.

Electron microscopic analysis (7, 8) and the solution of several crystal structures (4, 9, 10) have begun to provide a picture of the detailed conformational changes required for topoisomerization and have offered some insight to the mechanism of covalent catalysis and drug resistance. However, little is known about the regions of the protein required for coupling ATP-binding and hydrolysis to operation of the DNA gate.

In order to define these regions and to detect regions of the ATP-binding subunit that are involved in covalent catalysis, we designed a genetic screen to identify dominant-negative mutations in *parE*, encoding the ATP-binding subunit of *Escherichia coli* topoisomerase IV (Topo IV)‡ (11). We report in this and the accompanying articles (12, 13) the detailed analysis of the biochemical properties of Topo IV reconstituted with wild-type ParC (the DNA cleavage subunit; Refs. 14 and 15) and six mutant ParE subunits. All six mutant proteins were catalytically inactive and fell into one of two groups, those that were affected in either ATP binding or hydrolysis and which elicited hyper-DNA cleavage and those that were defective in formation of the covalent complex between ParC and DNA.

Here we describe the isolation of the mutant *parE* alleles, their phenotype when overexpressed *in vivo*, and the purification and initial characterization of the mutant ParE proteins. The accompanying articles (12, 13) describe the detailed characterization of the two different classes of mutant proteins.

**MATERIALS AND METHODS**

*Reagents, Enzymes, and DNA—Hydroxyamine, DAPI, and paraformaldehyde were from Sigma. Restriction enzymes and bacteriophage T4 DNA ligase were from New England Biolabs. *Pfu* polymerase was from Stratagene. SeaKem ME agarose was from FMC. Acrylamide was from Bio-Rad. Hybaid ECL nitrocellulose membrane and ECL-Western blotting detection reagents were from Amersham Pharmacia Biotech. Wild-type ParE and ParC were as described by Peng and Mariana (15). Polyclonal antisera against ParC and ParE were raised in rabbits. Goat anti-rabbit and goat anti-mouse IgG conjugated to horse-radish peroxidase was from Bio-Rad. Superhelical DNAs were purified by the alkaline lysis procedure (16), followed by equilibrium buoyant density gradient centrifugation in CsCl containing ethidium bromide. Plasmid pLex5BA was the kind gift of Walter Messer (Max-Planck-Institut, Berlin, Germany).

**Microbiological Techniques—DHSa, which was used to prepare all plasmid DNAs, was from Life Technologies, Inc. Cultures were grown in Luria broth or on Luria agar plates (17). When added, ampicillin was at 100 μg/ml, and IPTG was at 250 μg/ml. Competent cells were prepared by CaCl2 treatment (18) and were used for transformation as described in product information for Library Efficiency DHSa-competent cells (Life Technologies, Inc.).

To determine the efficiency of plating, cultures were grown at 37 °C to an *A*₀₀₀ of 0.5–1.0, diluted by 10⁻⁵ in L-broth, plated (0.1 ml), and grown

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‡ The abbreviations used are: Topo IV, topoisomerase IV; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole.
overnight on LB agar plates either in the absence or presence of IPTG.

Construction of Topoisomerase IV Expression Plasmid—Plasmid pLex5BA was used as the starting point for construction of the Topo IV expression plasmid. pLex5BA is derived from pLex1B (19). It contains the ColE1 origin of DNA replication, bla, lacI, and an expression cassette set by the promoter cassette in that the difference between expression levels in the presence and absence of IPTG is nearly 5000-fold.2 parE and parC were amplified by PCR from pET21a-parE and pET3e-parC (15), respectively, using the following combinations of primers: parE, N-terminal (5′-CGAATCTAGATCCGGAAATTAAAGCGGATCCGTCG-3′) and C-terminal (5′-CGACGATTGACTACCTTGATGAACTTATCGGATCCGTCG-3′); parC, N-terminal (ATCGATCCGGAGAATAGACTCTAGATCTATGAGCGATATGGCAGCGCGC-CTT-3′) and C-terminal (5′-GAGTCAGTAAAGCTTTTACTCTTCGCTGATACCCGCTGCT-3′). The parC PCR product was digested with EcoRI and BglII and inserted into EcoRI- and BamH1-digested pLex5BA DNA. The resulting recombinant plasmid DNA was digested with SalI and HindIII and ligated with SalI- and HindIII-digested parC PCR product to give the plasmid pLex5BA-parEC. The DNA sequence of the inserted regions was verified by automated DNA sequencing.

Preparation of a Mutagenized parE Library—5 μg of EcoRI-, BamH1-digested pLex5BA-parEC DNA from pLex5BA-parCE was treated with 0.4 M hydroxylamine for an average of 4 h at 65 °C and then dialyzed exhaustively at 4 °C against 10 mM Tris-HCl (pH 8.0 at 4 °C), 10 mM NaCl. The mutagenized fragment was religated with EcoRI- and BamH1-digested pLex5BA-parEC and transformed into DH5α. Roughly 150,000 ampicillin-resistant colonies were screened off the plates and combined. Plasmid DNA was then prepared from these cells directly.

ECL-Western Analysis—Cultures of C600(pLex5BA-parEC) were grown in L-broth (17) containing 0.4% glucose and 20 μg/ml thiamine at 37 °C to A600 = 0.24. IPTG was then added to the indicated concentrations, and growth was continued for 3 h at 37 °C. Aliquots (1 ml) were pelleted in a microcentrifuge and were resuspended in 0.65 ml of Laemmli SDS-PAGE loading buffer (20). Aliquots (25 μl uninduced and 5 μl induced) were subjected to SDS-PAGE through 10% gels (20). Gels were equilibrated in transfer buffer (47.8 mM Tris base, 386 mM glycine, and 0.03% SDS) for 20 min and then transferred to a Hybond-ECL membrane (18). The membranes were blocked overnight in 1× PBS, 0.1% Tween 20, and 5% nonfat milk; incubated with the appropriate primary antibodies (in blocking solution); washed in 1× PBS and 0.1% Tween 20; incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (in blocking solution); washed again; developed with ECL-Western blotting detection reagents as described by the manufacturer; and immediately exposed to x-ray film.

DAPI Staining and Fluorescent Microscopy— Cultures in either the presence or absence of IPTG of DH5α carrying pLex5BA, pLex5BA-parEC, or the appropriate pLex5BA derivative were seeded with a 1% inoculum of an overnight culture grown in the absence of IPTG and grown at 37 °C for 3 h either in the presence or absence of 250 μM IPTG, as indicated. Aliquots (0.4 ml) were pelleted in a microcentrifuge and resuspended in 4% paraformaldehyde to A600 = 0.75. Aliquots (50 μl) of cell suspension were spread on SuperFrost/Plus microscope slides (VWR Scientific) and the cells allowed to settle for 5 min. Excess cell suspension was removed and the slides allowed to air-dry. Slides were rinsed by dunking in tap water 10 times, air-dried, and stained by incubating 15 min in the dark in 0.05 μM DAPI in 1× PBS. Slides were rinsed by incubating 5 min in 1× PBS. After air-drying, one drop of fluorescence mounting medium was placed on each slide and a coverslip attached. Fluorescence photomicroscopy was done using an Olympus Vanox-T microscope. Images were recorded onto slide film, digitized using an AGFA Duoscan Scanner, and processed using Adobe Photoshop 4.0 software.

**Expression of Mutant ParE Proteins**—The expression vector pET21a (Novagen) was modified by replacing the DNA sequence between the XhoI and EcoRI cleavage sites with the sequence 5′-AATAATTTTTGTTAACAATTGGAAGGAGAC-3′ to give the plasmid pET21aΔXE. Mutated parE alleles were released from their respective pLex5BA-parEC DNAAs by digestion with EcoRI and SalI and ligated with EcoRI- and SalI-digested pET21a(AXE). Twelve-liter cultures of BL21(DE3) carrying the respective pET21a(AXE)-parE plasmids were grown to A600 = 0.37 at 37 °C. IPTG was added to 0.4 mM, and expression was induced for 3 h at 37 °C. The cells were chilled; harvested in mixed 25 mM Tris-HCl (pH 8.0 at 4 °C), 10 mM NaCl, and 0.2 mM/ml lysozyme. The suspension was incubated at 0 °C for 45 min and then heat-halted at 39 °C for 5–10 min. After chilling (all subse- quent steps at 4 °C), the centrifugation was performed in the Sorvall GSA rotor at 12,000 rpm for 1 h. The supernatant (fraction 1) was made 0.07% in Polymin P, stirred for 10 min, and the pellet was then cleared by centrifugation. Solid NH4SO4 was then added slowly to 50% saturation and the suspension stirred for 1 h. The pellet was collected by centrifugation and dissolved in a minimum volume of buffer A (50 mM Tris-HCl (pH 7.5 at 4 °C), 5 mM DTT, 1 mM EDTA, and 20% glycerol) to give fraction 2. Fraction 2 was dialyzed against buffer A overnight, adjusted to a conductivity equivalent to buffer A + 50 mM NaCl, and applied to an SP-Sepharose column (1 ml of packed gel bed/10 mg of protein in fraction 2) that had been equilibrated with buffer A + 50 mM NaCl. The column was washed with two volumes of equilibration buffer and then eluted with a 10-column volume gradient of 50–300 mM NaCl in buffer A. A fraction corresponding to a tenth the bed volume of the column was collected. ParE was pooled (fraction 3) on the basis of SDS-PAGE analysis. Fraction 3 was diluted to a conductivity equivalent to buffer A + 50 mM NaCl and applied to an heparin-agarose column (1 ml of packed gel bed/5 mg of protein in fraction 3) that had been equilibrated with buffer A + 50 mM NaCl. The column was washed with two column volumes of the equilibration buffer and eluted with a gradient of 50–500 mM NaCl in buffer A. Fractions equal to one-tenth the bed volume of the column were collected. ParE, eluting at 200 mM NaCl, was pooled (fraction 4) on the basis of SDS-PAGE analysis. Fraction 4 was applied directly to an hydroxyapatite column (1 ml of packed gel bed/3 mg of protein in fraction 4) that had been equilibrated with buffer A + 200 mM NaCl. The column was washed with 2 column volumes of the equilibration buffer and eluted with a 10-column volume gradient of 0–400 mM NaCl in buffer A. Fractions equal to one-tenth the bed volume of the column were collected. ParE was pooled (fraction 5) on the basis of SDS-PAGE analysis. Fraction 5 was dialyzed into storage buffer (50 mM Tris-HCl (pH 7.5 at 4 °C), 5 mM DTT, 1 mM EDTA, 50 mM NaCl, and 40% glycerol), divided into small aliquots, frozen in liquid N2, and stored at −80 °C. Typical yields of nuclease-free ParE proteins were 10–15 mg/liter.

Superhelical DNA Relaxation Assay—Topo IV was reconstituted by mixing either wild-type or mutant ParE in 10% molar excess over wild-type ParC in their storage buffer and incubating at 0 °C for 30 min to give a final concentration of Topo IV of about 50 μM. Reconstituted enzyme was then stored at −20 °C. Superhelical DNA reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5 at 30 °C), 6 mM MgCl2, 10 mM DTT, 1 mM EDTA, 50 μM ATP, 50 μM bovine serum albumin, 10 μM (molecules) superhelical pUCO DNA (plasmid DNA carrying E. coli oriC), and the indicated amounts of either wild-type or mutant Topo IV were incubated at 37 °C for 30 min. NaCl was then added to 200 mM and the incubation continued for 2 min. EDTA was then added to 50 mM and the incubation continued for an additional 5 min. SDS and proteinase K were then added to 1% and 0.5 mg/ml, respectively, and the incubation continued for an additional 15 min. One-fifth volume of a loading dye mixture was then added and the DNA products analyzed by electrophoresis through 1% agarose gels at 2 V/cm for 15 h using 50 mM Tris-HCl (pH 7.8 at 23 °C), 40 mM NaOAc, and 1 mM EDTA as the electrophoresis buffer. The gels were stained with ethidium bromide, and the images were recorded using a Bio-Rad Gel Doc imaging system.

**RESULTS AND DISCUSSION**

Isolation of Dominant-negative Alleles of parE—There are two general approaches to the use of amino acid mutagenesis in structure-function analysis. Site-specific amino acid replacements can be engineered in vitro either randomly or on the assumption that residues conserved between members of a multiprotein family will have important roles in enzyme function. On the other hand, a screen can be developed to identify amino acid residues necessary for function on the basis of the required action of the enzyme in vitro. The advantage of the
The latter approach is that no assumptions are made about the role of particular amino acid residues. We developed such a screen in order to identify amino acid residues of ParE that were required for catalysis by Topo IV.

Topo IV is required for cell viability (11, 21). However, screens that demand viability based on mutagenesis of the chromosomal copy of the gene often yield temperature-sensitive mutations. The underlying problem in that instance can often be a defect in correct folding of the polypeptide chain and not directly related to catalysis. To circumvent this issue, we used an approach designed to isolate alleles of \textit{parE} that were dominant-negative at high copy number compared with the wild-type chromosomal allele.

The basic approach was to mutagenize a plasmid-borne copy of \textit{parE} and select those alleles that killed the cell with high efficiency. In order to be able to recover the plasmid, tight regulation of the mutagenized \textit{parE} was required so that the cells carrying the plasmids could be replica-plated under conditions of both induced and repressed gene expression. To achieve such tight regulation, we used the pLex5BA plasmid developed by Messer and colleagues (19) (Fig. 1). This plasmid carries an expression promoter developed by H. Bujard where an early bacteriophage T7 promoter is modified to contain two \textit{lac} operators flanking the Pribnow box, oriented such that a loop would form between them when they were bound by \textit{lac} repressor. The difference in the levels of expression of the target gene from this promoter in the presence and absence of inducer is 5000-fold. The expression promoter is followed by a multicloning site and a transcription terminator.

Whereas expression from this plasmid is very tightly regulated, the Bujard promoter is a very efficient one, and we were concerned that the fully induced level of expression would be too high to be of value in our screen. We therefore introduced a spacer of 19 nucleotides between the Shine-Dalgarno sequence and the initiator ATG of \textit{parE} to reduce the level of expression. In addition, to ensure that the we would isolate alleles of \textit{parE} that were dominant-negative in the presence of active Topo IV, we also expressed \textit{parC} from the same transcript. To balance the level of expression of \textit{ParC} and \textit{ParE}, a spacer of similar length and nucleotide composition was inserted between the Shine-Dalgarno sequence and the initiator ATG for \textit{parC} as well (Fig. 1). The level of overexpression of Topo IV from

![FIG. 1. Schematic of the expression cassette of the pLex5BA-\textit{parEC} plasmid. The details of the construction are described in the text. The two nucleotide sequences denoted as \textit{N} are random sequences of identical G + C content. \textit{SD}, Shine-Dalgarno sequence.](image1)

![FIG. 2. Extent of overexpression of \textit{ParE} and \textit{ParC} from the pLex5BA-\textit{parEC} plasmid. C600(pLex5BA-\textit{parEC}) cells were grown at 37 °C to \(A_{600} = 0.24\) IPTG was added as indicated and the cells allowed to grow for an additional 3 h. Cells were then harvested and processed for ECL-Western analysis as described under “Materials and Methods.” Note that the material in the uninduced lane (0 IPTG) is derived from a 5-fold greater amount of cells than that in all other lanes. Several different exposures of the Western blot were analyzed by densitometry using a Molecular Dynamics Personal Densitometer SI to calculate the extent of overproduction.](image2)

![FIG. 3. Overexpression of the dominant-negative \textit{parE} alleles causes a \textit{par} phenotype. Cultures of C600 carrying either pLex5BA-\textit{parEC}, or pLex5BA-\textit{parEC} plasmids that carry the mutated \textit{parE} alleles were grown to \(A_{600} = 1.0\), IPTG was added to 250 \(\mu\text{M}\) as indicated and growth continued for an additional 3 h. Cells were then harvested, processed, and visualized by fluorescence microscopy as described under “Materials and Methods.”](image3)

### Table I

| Amino acid substitution | Mutation | Killing efficiency* |
|-------------------------|----------|---------------------|
| G110S                   | GGC → AGC | \(1 \times 10^4\) |
| S123L                   | TCG → TG  | \(2.6 \times 10^4\) |
| T201A                   | ACT → GCT | \(>1 \times 10^4\) |
| E418K                   | GAA → AAA | \(7.3 \times 10^4\) |
| G419D                   | GGT → GAT | \(1.5 \times 10^5\) |
| G442D                   | GGT → GAT | \(2.6 \times 10^4\) |

* The ratio of viable DH5α cells carrying the appropriate pLex5BA-\textit{parEC} plasmid plated in the absence and presence of 250 \(\mu\text{M}\) IPTG.
pLex5BA-parEC was determined, by quantitative Western blotting, to be roughly 70-fold greater than the endogenous level (Fig. 2).

To restrict mutagenesis to parE, the EcoRI/BamHI fragment of DNA that contained only parE was treated with hydroxylamine. After religation, a plasmid library was prepared by passage of the recombinant DNA through DH5a. This library was then retransformed into DH5a, plated on LB agar, and grown at 37 °C. Colonies were replica-plated onto LB agar containing 250 μg IPTG. Isolates that did not grow on IPTG were then grown in suspension in the presence of IPTG, and a relative killing efficiency was determined by plating on LB agar in either the presence or absence of IPTG. Only those isolates that exhibited a killing efficiency of $>10^4$ (without IPTG/with IPTG) were investigated further.

parE alleles carrying nonsense mutations were eliminated by determining the size of the expressed mutant ParE by SDS-PAGE and Western blotting. Those that expressed full-length protein were then sequenced to determine the position and nature of the mutation present. Six mutant ParE proteins were chosen for subsequent biochemical characterization (Table I). All except one of these mutations occurred at amino acid residues that were conserved among type II topoisomerases (6).

Gly$^{110}$ corresponds to Gly$^{114}$ of GyrB and, based on the crystal structure of the N-terminal fragment of GyrB complexed with ATP (9), is known to contribute to the stabilization of the $\text{Mg}^{2+}$-ATP complex. Gly$^{110}$ and Ser$^{123}$ are conserved in all type II topoisomerases except Mycobacterium leprae, where the corresponding residues are a Ser and Ala, respectively. Glu$^{418}$ and Gly$^{419}$ are part of the EGDSA motif that is conserved in all type II topoisomerases. Gly$^{442}$ is part of the PLRGIKN motif and is conserved in all type II topoisomerases except Caenorhabditis elegans 2C, where the corresponding residue is an Arg. On the other hand, Thr$^{201}$ is not conserved at all. Some of these amino acid residues have been mutated in other studies. Those results will be discussed in comparison to our observations as reported in the accompanying articles (12, 13).

Defects in Topo IV function should result in a par phenotype in vivo, where the chromosomes continue to replicate but cannot be decatenated. This results in a long, filamented cell with a large nucleoid in the center. To ensure that we were focused on amino acid residues that were involved in catalytic function, we examined the phenotype induced when the mutant parE alleles were expressed from the corresponding pLex5BA-parEC plasmids (Fig. 3). After 3 h of induction, expression of all the mutant ParE proteins caused a par phenotype. There was a striking difference between the effect of the ParE T201 Topo IV and all the others.

C600 carrying pLex5BA-parCE appeared essentially wild-type in the absence of IPTG (Fig. 3A). Overexpression of wild-type Topo IV caused the cells to become larger, suggesting a delay in cell division. However, the majority of the cells observed were typical dividing cells with two nucleoids. With the exception of the ParE T201A Topo IV, overexpression of all the mutant Topo IVs caused a classical par phenotype. On the other hand, when ParE T201A Topo IV was overexpressed, the cells became elongated and filamented, but there was no nu-

![Image 1](https://example.com/image1.png)

**FIG. 4.** SDS-PAGE analysis of the purified mutant ParE proteins. One microgram of the indicated ParE proteins were analyzed by SDS-PAGE through a 10% gel. The gel was stained with Coomassie Brilliant Blue. WT, wild-type.
cleoid of any size present. Instead, a diffuse DAP1 staining was evident. This suggested that overproduction of this mutant Topo IV actually causes degradation of the DNA. This proved to be the case.

**Purification of Mutant ParE Proteins and Initial Characterization of Enzymatic Activity**—DNA fragments carrying the mutant parE alleles were excised from their respective pLex5BA-parEC plasmids by digestion with EcoRI and SalI and inserted into similarly digested pET21aΔXE plasmid DNA for overexpression and purification. Typically, 12-liter cultures of induced BL21(DE3) cells were grown for purification. Soluble lysates were prepared, nucleic acids were removed by treatment with Polymin P, and the protein was concentrated by precipitation with (NH₄)₂SO₄. Mutant ParE proteins were then purified by subsequent chromatography on Q-Sepharose FF, heparin-agarose, and hydroxylapatite. The purified preparations (Fig. 4) were free of any detectable single- or double-stranded DNA endo- and exonuclease (data not shown).

Topo IV was reconstituted with wild-type ParC and the mutant ParE proteins. The ability of the mutant Topo IV proteins to relax superhelical DNA was then assessed (Fig. 5). Approximately 0.15 pmol of wild-type Topo IV could relax 0.2 pmol of superhelical DNA in 30 min at 37 °C. The mutant Topo IV proteins displayed a spectrum of activity. The ParE E418K and ParE G442D Topo IV proteins were completely inactive, even when the reaction mixture contained 20 pmol of tetramer. The ParE G419D Topo IV could catenate DNA at the highest concentration and appeared to be about 4-fold less active than the wild-type in this reaction. However, it was clearly 30–50-fold less active than the wild-type in superhelical DNA relaxation.

The ParE G110S, ParE S123L, and ParE T201A enzymes all exhibited hyper-DNA cleavage. In processing the DNA for gel electrophoresis in the assays shown, enough NaCl is added to prevent re-binding of any Topo IV that dissociates from the DNA, in addition, EDTA is also added to allow re-sealing of any covalent Topo IV-DNA complexes before SDS and proteinase K are added. Under these conditions for terminating the reaction, no DNA cleavage is observed in this assay with concentrations of the wild-type enzyme sufficient to relax all the DNA. Thus, the observation of hyper-DNA cleavage suggests that a disruption of the normal cycle of cleavage and religation has occurred.

In the accompanying articles (12, 13), we investigate the properties of these mutant proteins in detail. Interestingly, all three of the mutant Topo IV proteins that exhibit hyper DNA cleavage are unable to hydrolyze ATP. Thus, these mutations appear to have uncoupled the coordination required for linkage of the cycle of ATP binding and hydrolysis to the DNA cleavage and religation cycle. The other three mutations effect the ability of the enzyme to form the covalent intermediate, thus clearly indicating that there are regions in ParE that must undergo significant conformational rearrangement to participate in the chemistry of linking DNA to the active site Tyr residue of ParC.

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