Mutational Analysis of Escherichia coli Topoisomerase IV

II. ATPase NEGATIVE MUTANTS OF ParE INDUCE HYPER-DNA CLEAVAGE*

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ParE is the ATP-binding subunit of topoisomerase IV (Topo IV). During topoisomerization, the ATP-binding and hydrolysis cycle must be coordinated with the cycle of DNA cleavage and religation. We have isolated three dominant-negative mutant alleles of parE that encode ParE proteins that fail to hydrolyze ATP when reconstituted with ParC to form Topo IV. ParE G110S Topo IV and ParE S123L Topo IV failed to bind ATP at all, whereas ParE T201A could bind ATP. All three mutant Topo IV proteins exhibited an elevated level of spontaneous DNA cleavage that could be associated with a decreased rate of DNA resealing. In ParE T201A Topo IV, this defect appeared to result from an increased likelihood that the tetrameric enzyme would fall apart after DNA cleavage. Thus, while ATP is not required for DNA cleavage, the properties of these mutant enzymes suggest that ATP-hydrolysis informs DNA religation.

Type II topoisomerases couple the energy of ATP hydrolysis to alter the linking number of DNA (1–3). To do so, these enzymes must execute an ordered sequence of conformational changes and chemical reactions: trapping a segment of DNA, passage of the trapped segment through a transient double-strand break in another DNA segment, release of the passed DNA segment from the interior of the enzyme, and resetting of the enzyme for another round of catalysis. Thus, these enzymes have two catalytic activities: ATP binding and hydrolysis, and DNA cleavage and religation.

Roca and Wang (4) clearly demonstrated that one role for ATP binding and hydrolysis is the operation of a protein clamp on the enzyme to trap the segment of DNA (the T segment) to be transported through the transient double-strand break (the G segment). Use of such a clamp is probably not an absolute necessity for topoisomerase activity, but it clearly increases the catalytic efficiency of the enzyme by orders of magnitude. ATP binding is sufficient to close the clamp, whereas hydrolysis of ATP is presumed to reset it in the open position, although this has not been demonstrated directly.

DNA cleavage and religation functions to open and close the DNA gate through which the T segment must be passed in order to effect changes in DNA topology (1–3). Both DNA cleavage and religation and ATP binding and hydrolysis are manifest in the absence of the other activity, although DNA binding appears to stimulate ATP hydrolysis (1–3). Yet, it is clear that, during topoisomerization, the two reactions must be coordinated. ATP hydrolysis without DNA transport would render the enzyme very inefficient, allowing the captured T segment to escape without alteration of DNA topology.

Little is known about the manner in which these reactions are coordinated and the amino acid residues important for this crucial linking of enzymatic functions. As a result of a screen for dominant-negative mutations of parE (5), we have isolated and characterized three mutant ParE proteins defective in either ATP binding or ATP hydrolysis when reconstituted with ParC to form Escherichia coli topoisomerase IV (Topo IV). All three of the mutant Topo IV proteins exhibit hyper-DNA cleavage in the absence of ATP, suggesting that the mutations have induced a conformation that disrupts communication between the DNA cleavage and ATP hydrolysis domains. In the case of ParE T201A Topo IV, which can bind but not hydrolyze ATP, hyper-DNA cleavage can be correlated with a decrease in the rate of resealing of the cleaved DNA and an increased chance that the tetrameric enzyme will fall apart after cleavage, leaving ParC linked covalently to the DNA. This suggests that, under normal circumstances, ATP hydrolysis is required for the DNA gate to close.

MATERIALS AND METHODS

Reagents, Enzymes, and DNAs—Kinetoplast DNA from Crithidia fasciculata (kDNA) was from TopoGEN, Inc. (Columbus, OH). [2,8-3H]AMP-PNP, tetrasodium salt (20.5 Ci/mmol) was from Moravek Biochemicals, Inc. (Brea, CA). All other reagents, enzymes, and DNAs were as detailed in the accompanying article (5).

Superhelical DNA Relaxation Assay—Assay was performed as described in the accompanying article (5).

Decatenation of kDNA—Standard reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5 at 37 °C), 6 mM MgCl₂, 10 mM dithiothreitol, 100 mM potassium glutamate, 50 µg/ml bovine serum albumin, 1 mM ATP, 9 mts (as monomeric minicircles) kDNA, and the indicated amounts of either wild-type or mutant Topo IV proteins were incubated at 37 °C for 30 min. EDTA was then added to a final concentration of 50 mM and the incubation continued an additional 2 min. SDS and proteinase K were then added to final concentrations of 1% and 5 µg/ml, respectively, and the incubation continued an additional 15 min. Gel electrophoresis tracking dyes were then added and the DNA products electrophoresed through 1.2% agarose (SeaKem ME, FMC) gels at 2 V/cm for 15 h at room temperature using 50 mM Tris-HCl (pH 7.8 at 23 °C), 40 mM NaOAc, and 1 mM EDTA as the electrophoresis buffer. Gels were stained with ethidium bromide, and images were recorded using a Bio-Rad GelDoc imaging system.

ATPase Assay—Standard reaction mixtures (20 µl) containing 40 mM Tris-HCl (pH 7.5 at 30 °C), 6 mM MgCl₂, 10 mM dithiothreitol, 20 mM KCl, 50 µg/ml bovine serum albumin, 1 µM spermine-HCl, 0.4 µg of pBSM13 DNA (Stratagene), 0.5 mM ATP, 1 µCi of [γ-32P]ATP, and the indicated amounts of either wild-type or mutant Topo IV proteins were incubated at 37 °C for 15 min. The amount of 32P generated was then determined by the method of Conway and Lipmann (6).

ATP-binding Assay—Standard reaction mixtures (10 µl) containing 50 mM Tris-HCl (pH 7.5 at 30 °C), 20 mM KCl, 5 mM dithiothreitol, 12

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1 The abbreviations used are: Topo IV, topoisomerase IV; AMP-PNP, 5'-adenylyl imidodiphosphate; kDNA, kinetoplast DNA from C. fasciculata; PAGE, polyacrylamide gel electrophoresis.
mm MgCl₂, 50 μg/ml bovine serum albumin, 1 mM [³²H]AMP-P(NH)P (56 cpm/pmole), and either 5.3 μM wild-type or mutant Topo IV proteins were incubated at 37 °C for 15 min, diluted with 90 μl of washing buffer (reaction buffer without the bovine serum albumin, dithiothreitol, and [³²H]AMP-P(NH)P, but with 100 μM cold AMP-P(NH)P), filtered through nitrocellulose filters (Millipore, HAWP), and then rinsed three times with washing buffer (1 ml each wash). Filters were then dried and the radioactivity retained determined by liquid scintillation spectrometry. Filters were soaked in 0.5 M KOH for 20 min, neutralized in 0.1 M Tris-HCl (pH 7.5 at 23 °C) for 40 min, and then equilibrated in binding buffer for 30 min before use.

**Covalent Complex Formation**—Standard reaction mixtures (20 μl) containing 40 mM Tris-HCl (pH 7.5 at 37 °C), 6 mM MgCl₂, 10 mM dithiothreitol, 20 mM KCl, 100 μg/ml bovine serum albumin, 1 mM spermidine-HCl, 1 mM ATP, 0.27 μg (130 fmol molecules) of [³²P]pBSM13 DNA (labeled by nick translation to 2 × 10⁶ cpm/μg), and 0.16 μM wild-type or mutant Topo IV proteins were incubated at 37 °C for 3 min. Norfloxacin was then added to 250 μM and the incubation continued for an additional 20 min. One-fifth volume of Laemmli (7) SDS-PAGE loading dye was then added and the reaction products analyzed by SDS-PAGE through 10% gels (7). The gel was dried and autoradiographed.

**DNA-binding Assay**—Binding of either wild-type or mutant Topo IV proteins to the 50-mer double-stranded oligonucleotide T450, composed of a defined Topo IV binding sequence, was as described by Marians and Hissia (8).

**ParE Cross-linking**—Standard reaction mixtures (15 μl) containing 50 mM Hepes-KOH (pH 8.0 at 23 °C), 100 mM KCl, 4 mM dithiothreitol, 4 mM MgCl₂, 1 mM AMP-P(NH)P, where indicated, and 2 μM either wild-type or mutant ParE proteins were incubated at 23 °C for 30 min. Dimethyl suberimidate di-HCl (Pierce) was then added to 4 mg/ml (from a 34 mg/ml stock solution prepared in triethanolamine) and the incubation continued for an additional 30 min. The reaction was quenched by the addition of glycerine to 62 mM and the reaction products analyzed by SDS-PAGE through 9% gels. The gels were stained with Coomassie Brilliant Blue and photographed.

**Gel Filtration of Topo IV**—Wild-type or mutant Topo IV (25 μl of 8–20 μM) was gel filtered through a FPLC Superose 6 column (4 ml) at 0.2 ml/min using 50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 0.5 mM dithiothreitol, 150 mM NaCl, and 10% glycerol as the column buffer. Fractions (100 μl) were collected from the time of injection. Aliquots (10 μl) were analyzed by SDS-PAGE through 10% gels. The gels were stained with Coomassie Brilliant Blue and photographed.

**RESULTS**

Hyper-DNA Cleavage by mutant Topo IV Proteins Does Not Require ATP.—In the first article in this series (5), we described the isolation of dominant-negative parE mutants and the initial characterization of the superhelical DNA relaxation activity of Topo IVs reconstituted with the mutant ParEs and ParC. Three of these mutant proteins, ParE G110S, ParE S123L, and ParE T201A Topo IVs, exhibited hyper-DNA cleavage under conditions where the reaction was terminated and processed for agarose gel electrophoretic analysis as described under “Materials and Methods.”

![Fig. 1. Mutant Topo IV proteins exhibit hyper-DNA cleavage.](image)

The indicated Topo IV proteins (20 pmol) were incubated in standard DNA relaxation reaction mixtures for 30 min at 37 °C. The reactions were terminated and processed for agarose gel electrophoretic analysis as described under “Materials and Methods.”

![Results](image)

Ensure that any Topo IV bound to the DNA in an open complex resealed the cleaved DNA and dissociated. Only then was SDS added to denature the protein. The observation of hyper-DNA cleavage under these conditions suggests a corruption of the normal cleavage-religation equilibrium.

The three mutations under investigation were all in the ATP-binding subunit of Topo IV. Based on the crystal structure of a fragment of GyrB bound to ATP (9), at least one of the targeted amino acid residues, Gly⁹¹⁰, was known to be involved in ATP-binding. Therefore, DNA cleavage by the wild-type and mutant Topo IVs was compared in the presence and absence of ATP (Fig. 1). In the presence of ATP, the wild-type enzyme catedenated the DNA, as expected at this high concentration (1 μM). As noted in the accompanying article (5), neither the ParE G110S, ParE S123L, nor ParE T201A mutant Topo IVs could cationate the DNA, as expected at this high concentration (1 μM). As noted in the accompanying article (5), neither the ParE G110S, ParE S123L, nor ParE T201A mutant Topo IVs could cationate the DNA, and they all produced a high level of cleaved DNA. The situation was similar in the absence of ATP. Under these conditions, a comparison could be made to the wild type. Densitometric analysis (Table I) showed that in the absence of ATP, the ParE G110S and ParE S123L mutant enzymes produced about 2- and 3-fold more cleaved DNA, respectively, than the wild type. The ParE T201A Topo IV produced at least 6-fold more cleaved DNA than the wild type. This is probably an underestimate, because all of the DNA was cleaved by this protein. Little modulation in either the presence or absence of ATP of the extent of cleavage by the mutant enzymes was evident (Table I). Cleavage was dependent on the presence of both subunits for all the enzymes (data not shown); thus, it was unlikely to be the result of nuclease contamination.

If the observed DNA cleavage was a result of corruption of the cleavage-religation equilibrium, then it was possible that the mutations could effect covalent complex formation. This was measured directly. Wild-type and mutant Topo IVs were bound to [³²P]DNA at about a 25-fold molar excess of enzyme. Norfloxacin was added to shift the enzyme-DNA complex to the open form, where ParC is covalently bound to DNA and then the excess DNA was removed by digestion with DNase I. This results in transfer of the label to ParC as a result of a few nucleotides of DNA remaining covalently attached to the protein. The results were analyzed by SDS-PAGE (Fig. 2). All the
mutant Topo IVs were capable of forming covalent complex as well as the wild type. Thus, the underlying defect that caused the hyper-DNA cleavage had to be in another activity of the enzyme. It should be noted that, in the label transfer assay (Fig. 2), the ratio of Topo IV to DNA is much lower than it is in the experiment shown in Fig. 1. This different enzyme-to-substrate ratio accounts for the increased cleavage activity of ParE T201A Topo IV compared with the other two mutant enzymes in Fig. 1.

The Mutant Topo IVs Are Defective in ATP Binding and Hydrolysis—ATP hydrolysis in the presence of DNA was measured for the wild-type and mutant Topo IVs as a function of enzyme concentration (Fig. 3). All three of the mutant proteins were defective in their ability to hydrolyze ATP. The extent of the defect was variable, with the specific activities of the ParE G110S, ParE S123L, and ParE T201A Topo IVs being reduced to one-fifteenth, one-fortieth, and one-twentieth, respectively, that of the wild-type enzyme. This defect could, of course, be the result of either the inability of the enzyme to bind ATP or a disruption of the chemical steps required for hydrolysis. To distinguish between these possibilities, the ability of the mutant and wild-type enzymes to bind ATP was evaluated.

A nitrocellulose filter binding protocol was used to assess binding of [3H]AMP-P(NH)P to the various Topo IV proteins (Table II). The nonhydrolyzable analog of ATP was used so that stable binding could be observed without the complications of concomitant ATP hydrolysis. This analysis showed clearly that the mutant Topo IVs fell into one of two groups. Neither ParE G110S nor ParE S123L Topo IV could bind significant amounts of [3H]AMP-P(NH)P. This is consistent with their reduced ability to hydrolyze ATP. On the other hand, ParE T201A Topo IV bound [3H]AMP-P(NH)P as well as the wild-type enzyme. The reason for the failure of this enzyme to hydrolyze ATP is not obvious at this time. Some possibilities are considered under “Discussion.”

After treatment with dimethyl suberimidate, both the wild-type and T201A ParE proteins produced a new AMP-P(NH)P-dependent species that migrated with variable mobility in polyacrylamide gels. Mobility of this band was dependent on the duration of electrophoresis. When the gels were electrophoresed for only 1.5 h, the band migrated at 150 kDa (data not shown). In the gel shown, electrophoresis was for 3 h and the band migrated at about 220 kDa. A dimer of ParE is predicted to be 140.4 kDa. Thus, it is conceivable that the cross-linked material is a trimer. However, because it migrates at shorter times of electrophoresis, it is most likely the dimer species. The aberrant migration at longer electrophoresis times is currently unexplained. Neither the G110S nor S123L ParE could be cross-linked to yield the slowly moving species (Fig. 4). In all cases, the monomer bands became broadened and a new species migrating somewhat more slowly than the monomer band became visible compared with untreated samples. We suspect that this is a result of internal cross-linking of the protein.
Roca and Wang (4) demonstrated that closing of the ATP-dependent clamp and capture of a T segment was sufficient to allow the yeast type II topoisomerase to catenate DNA rings. Thus, our observation that the T201A ParE could dimerize suggested that Topo IV reconstituted with this subunit should be capable of some aspect of topoisomerase activity. We presumed could not observe any activity in the superhelical DNA relaxation assay because of the extensive DNA cleavage at high levels of ParE T210A Topo IV. The ability of this enzyme to decatenate kDNA was therefore compared with that of the wild-type and the other two mutant enzymes.

Wild-type Topo IV was, as has been noted before (10, 11), a very efficient decatenating enzyme. At a concentration of 40 μM ATP, which is the optimum for this reaction with the wild-type enzyme (12), 50% of the kDNA was decatenated in a 15 min reaction at a ratio of one Topo IV molecule to 20 monomers of enzyme (12), 50% of the kDNA was decatenated in a 15 min reaction at a ratio of one Topo IV molecule to 20 monomers of enzyme (12). Under identical conditions, none of the three mutant Topo IVs displayed any decatenation activity (data not shown). We could, however, detect decatenation activity when we increased the concentration of the mutant enzymes significantly and also increased the ATP concentration to 1 mM.

Under conditions of increased ATP concentration, a roughly 1200-fold greater concentration of ParE T201A Topo IV was required to observe a similar extent of decatenation as catalyzed by the wild-type protein (Fig. 5D). The ParE G110S and ParE S123L Topo IVs exhibited much less activity, although some decatenation could also be observed (Fig. 5B and C). This suggests that at least the ParE T201A Topo IV is unlikely to have a defect in strand transport, per se. The requirement for much higher concentrations presumably reflects the fact that once the ATP-dependent clamp closes on this mutant enzyme, it cannot reopen. Thus, if a T segment has not been trapped upon closing, that particular molecule is rendered inactive.

The Mutant Topo IVs Exhibit Spontaneous DNA Cleavage—The data described thus far clearly associate the inactivity of the mutant Topo IVs to their inability to hydrolyze ATP. However, they do not reveal the underlying reason for the hyper-DNA cleavage that was observed. We therefore examined the cleavage reaction in more detail to determine whether cleavage was spontaneous and whether ParC remained bound to the cleaved DNA.

Wild-type and mutant Topo IVs were incubated with superhelical DNA either in the presence or absence of ATP or in the presence of AMP-P(NH)P. The reactions were terminated three different ways, either by the addition of (i) only gel tracking dyes, (ii) SDS, or (iii) SDS and proteinase K. Samples terminated by methods (ii) and (iii) were incubated an additional 15 min after these final additions. Reaction products were then analyzed by agarose gel electrophoresis. Cleaved DNA evident in reactions terminated by method (i) presumably represents spontaneous DNA cleavage, i.e. DNA that has been released from the enzyme while it is in the open conformation without denaturation of the protein. The pattern evident with reactions terminated with SDS gives an approximation of how much Topo IV is covalently bound to the DNA. Comparison of the DNA products of reactions terminated with only tracking dye to those terminated with SDS should give a clear indication of whether there is protein bound to the DNA under the former condition. Covalently bound protein will be evident by the appearance of a smear of DNA in the lane from the position of the linear to the gel origin. Finally, the extent of cleavage observed in reactions terminated with both SDS and proteinase K reflects the total amount of cleavage, the sum of both the spontaneous cleavage and cleavage resulting from denaturing enzyme in the open complex.

The wild type did not exhibit any spontaneous cleavage (Fig. 6, A, B, and C, lane 2). This indicates that normally the frequency of spontaneous cleavage is very low, as would be expected because generation of such cleaved DNA in the cell would be a lethal event. On the other hand, the ParE G110S (Fig. 6, A, B, and C, lane 5) and ParE S123L (Fig. 6, A, B, and C, lane 8) Topo IVs exhibited spontaneous cleavage, with the ParE S123L enzyme showing a higher level. Neither of these mutant enzymes showed the expected smearing of DNA in the lane if there was protein covalently bound to the cleaved DNA. The type of smearing expected is evident in those reactions that were terminated with SDS (Fig. 6, A, B, and C, lane 6 for ParE G110S Topo IV and lane 9 for ParE S123L Topo IV).

ParE T201A Topo IV exhibited a different behavior. It clearly cleaved DNA spontaneously (Fig. 6, A, B, and C, lane 11), but in this case, ParC apparently remained bound to the cleaved DNA as the patterns were similar to those observed for reactions terminated with SDS (Fig. 6, A, B, and C, lane 12).

ATP appeared to inhibit total cleavage by both the ParE G110S (Fig. 6, A, B, and C, lane 7) and ParE S123L (Fig. 6, A, B, and C, lane 10) Topo IVs. On the other hand, AMP-P(NH)P appeared to stimulate total cleavage by the ParE T201A Topo IV. The effect of ATP on cleavage by the wild-type enzyme is more difficult to estimate because catenated DNA can also account for disappearance of the substrate (Fig. 6A, lane 3).
This issue of whether protein remained bound to DNA cleaved spontaneously by the ParE T201A Topo IV was examined in another manner. Topo IV can bind to a 50-nucleotide-long duplex oligonucleotide that is composed of a palindromic defined DNA-binding site (10). If ParC was remaining covalently bound to DNA cleaved spontaneously by this mutant enzyme, binding to the 50-mer should be stimulated significantly compared with the wild-type and the other two mutant proteins. This proved to be the case (Fig. 7).

The ParE G110S and ParE S123L Topo IVs bound the 50-mer to an extent similar to that for the wild-type protein, with about 20–30% of the DNA retained on the filters at a 2 mM concentration of enzyme. Binding by the ParE T201A Topo IV was significantly better, with nearly 80% of the DNA bound at one-eighth that concentration. This rough comparison suggests that the ParE T201A Topo IV binds the 50-mer with an efficiency at least 24-fold better than any of the other enzymes. This is consistent with ParC remaining bound to the DNA after spontaneous cleavage by this enzyme and not the others.

The Mutant Topo IVs Are Defective in Resealing DNA after Cleavage—The observation that the mutant Topo IVs exhibited spontaneous DNA cleavage suggested that they were defective in resealing the DNA after cleavage. To address this, we developed an assay to measure the rate of resealing. The enzymes were allowed to establish a cleavage-religation equilibrium with the DNA at 15 °C. The lowered temperature was used to slow the resealing reaction in order to make it easier to measure the rate of reaction. NaCl was then added to the reaction mixture to a concentration of 300 mM. This is sufficient to prevent rebinding to the DNA of any enzyme that dissociates. Resealing of the cleaved DNA (both form II and form III) was then followed kinetically (Fig. 8).

Even under the conditions of reduced temperature, the wild type resealed the DNA quickly, with a $t_{1/2}$ of about 1 min (Fig. 8, A and E). The ParE G110S (Fig. 8B) and ParE S123L (Fig. 8C) Topo IVs both had rates of resealing that were reduced by one-half to one-quarter that of the wild type with $t_{1/2}$ values of about 3.9 and 2.8 min, respectively (Fig. 8E). The ParE T201A Topo IV was extremely defective in resealing (Fig. 8, D and E). A $t_{1/2}$ could not be calculated for this data because of the difficulty in obtaining a good curve fit, but it is likely to be greater than 10-fold that of the wild type.

Thus, all three mutant proteins exhibited a qualitatively similar defect in that they did not reseal cleaved DNA as fast as the wild type. However, the magnitude of this defect was significantly greater for the ParE T201A Topo IV than for the other two mutant proteins. Significantly, it also appeared that ParC remained covalently bound to the ends of the DNA cleaved by the ParE T201A enzyme, but that this was not the case for the other two mutant proteins. This suggested that the ParE T201A Topo IV tetramer was actually falling apart after DNA cleavage. If this were the case, one would expect that, in general, the stability of this tetramer would be low and that this would be apparent by gel filtration. This proved to be the case.

The mutant and wild-type Topo IVs were reconstituted with a 10% excess of the ParE subunit and gel-filtered through a FPLC Superose 6 column. The distribution of ParE and ParC was followed by SDS-PAGE (Fig. 9). The patterns for the ParE

![Fig. 5. At very high concentrations, the ParE T201A Topo IV, but neither the ParE G110S nor ParE S123L Topo IVs, can decatenate kDNA. Standard decatenation assays were performed with the indicated Topo IV proteins, and the reactions were analyzed by agarose gel electrophoresis as described under “Materials and Methods.” Note that the concentrations for the wild-type protein are nanomolar, whereas that of the three mutant proteins is micromolar.](image)

![Fig. 6. Analysis of the nature of the hyper-DNA cleavage mediated by the mutant Topo IV proteins. Either the wild-type (20 pmol), ParE G110S (40 pmol), ParE S123L (40 pmol), or ParE T201A (10 pmol) Topo IVs were incubated in standard superhelical DNA relaxation reaction mixtures either in the absence of ATP (panel C) or in the presence of either ATP (panel A) or AMP-P(NH)P (panel B) at 37 °C for 30 min as described under “Materials and Methods.” The reaction mixtures were either loaded directly (lanes 2, 5, 8, and 11), made 1% in SDS (lanes 3, 6, 9, and 12) and incubated an additional 15 min at 37 °C, or made 1% in SDS and 200 μg/ml in proteinase K and incubated an additional 15 min at 37 °C (lanes 4, 7, 10, and 13) before analyzing the reaction products by agarose gel electrophoresis.](image)
G110S and ParE S123L Topo IVs were identical to that of the wild type. A clear shift in the distribution of both ParC and ParE was observed, and the stoichiometry of the two subunits in the peak fractions was 1:1 (as determined by densitometry of the dried gel; data not shown). In contrast, the ParE T201A Topo IV clearly did not form a stable tetramer. In this profile, ParC and ParE clearly eluted at different positions. Thus, the tetramer formed by this mutant ParE with ParC is considerably less stable than the ones formed by the wild-type and the other two mutant proteins.

**DISCUSSION**

In order to alter the topology of DNA, type II topoisomerases must execute a complex and ordered series of chemical reactions and conformational changes. To do so, they have coupled the energy of ATP binding and hydrolysis to transient cleavage and religation of double-stranded DNA. In bacteria, the ATP-hydrolyzing domains and cleavage and religation domains are in different subunits of heterotetrameric enzymes, whereas in eukaryotes, they are joined together on one large polypeptide that forms a homodimer. The organization of the eukaryotic enzymes is such that they correspond to joining the prokaryotic ATP-binding and DNA cleavage and religation subunits together as N- and C-terminal domains of one polypeptide. Amino acid sequence conservation between the prokaryotic and eukaryotic enzymes is very strong (13, 14), suggesting that they operate by similar, if not identical, mechanisms.

There has been considerable effort devoted to elucidating the mechanism of action of this family of proteins. Biochemical analyses have outlined the basic steps required for these enzymes to pass one helix of DNA through another (1–3). The role of ATP binding and hydrolysis is less well defined. Although it has been demonstrated that ATP binding causes dimerization of the ATP-binding domain and that this, in turn, raises the probability that a segment of DNA will be trapped for transport to the interior of the enzyme through a transient double-strand break in another DNA segment (4), how the ATP-mediated steps are coupled to the large conformational changes required for cleavage, strand passage, and religation remains elusive. In fact, at least with DNA gyrase, ATP is not required for relaxation of negatively supercoiled DNA (15, 16). Our characterization of Topo IV proteins consisting of ParC and mutant ParE subunits suggests that both ATP binding and DNA cleavage can trigger conformational changes that are sensed by distant regions of the enzyme.

In a screen for dominant-negative mutations in *parE* (5), we isolated three mutant alleles that encoded ParE proteins that were defective in their ATPase activity when reconstituted with ParC to form Topo IV. Of these, two, ParE G110S and ParE S123L Topo IVs were defective in binding ATP, whereas the other, ParE T201A, could bind ATP as well as the wild type, but was defective in hydrolysis.

None of these mutant proteins could relax negatively supercoiled DNA, although ParE T201A Topo IV could decatenate *Crithidia* kinetoplast DNA at concentrations 1200-fold greater than that required for the wild type. It is possible that this is a result of contamination with chromosomally encoded wild-type ParE in the preparation of ParE T201A. However, we think that this is unlikely because the other two mutant ParE proteins were purified in an identical fashion from identical amounts of starting material, yet did not exhibit the same,
extremely low decatenation activity. In addition, the activity of the ParE T201A Topo IV is consistent with the demonstration that ParE T201A could be cross-linked as a dimer, whereas the other two mutant proteins could not. In a rough sense, this 1200-fold difference in the amount of protein required for decatenation by ParE T201A Topo IV compared with the wild type gives a minimum estimate for the gain in catalytic efficiency afforded by active trapping of the T segment by the ATP-dependent clamp.

Remarkably, all three mutant Topo IVs exhibited a significantly higher frequency of spontaneous DNA cleavage compared with the wild type. There was a significant difference between the extent of cleavage and the disposition of ParC that correlated with the difference in the ability of the enzymes to bind ATP. The ParE G110S and ParE S123L Topo IVs showed similar levels of cleavage and there was no evidence of ParC remaining bound to the 5′-end of the DNA break. On the other hand, the ParE T201A Topo IV showed much higher levels of cleavage and ParC remained attached to the 5′-end of the DNA break. It could be demonstrated that, in all cases, there was a defect in the rate of resealing of the transient double-strand break mediated by the enzyme, and in the case of the ParE T201A enzyme, it appeared as though the protein was falling apart after DNA cleavage. Interestingly, this property of increased spontaneous DNA cleavage associated with mutant type II topoisomerases defective in ATP hydrolysis has not been noted before.

Several amino acid replacements have been engineered in type II topoisomerases resulting in enzymes defective for ATP hydrolysis. The K359N and K359E (Lys337 in DNA gyrase and Lys334 in ParE) amino acid substitutions in the Drosophila enzyme both inactivate the ATPase and superhelical DNA relaxation activities of the enzyme; however, DNA cleavage was unaffected (17). A similar mutation in GyrB, K337Q, reduces ATP-binding, ATP hydrolysis, and DNA supercoiling to one-
half, one-fiftieth, and less than one-hundredth of the levels of the wild-type DNA gyrase (18). No significant effect on quinolone-induced DNA cleavage was noted. Various replacements of GyrB Lys\textsuperscript{101} all showed negligible ATP binding and hydrolysis as well as 2–3-log reductions in supercoiling activity (19). Again, no significant effect on quinolone-induced DNA cleavage was noted. Based on the crystal structure of the N-terminal domain of GyrB bound to AMP-PNP\textsubscript{H} (9), the amino acid residues replaced in these studies all contact the ATP.

The existing GyrB structure (amino acid residues 2–392) can be divided into two domains (Fig. 10). The first domain (residues 2–220, white in Fig. 10) is composed of a mixed eight-stranded \( \beta \)-sheet backed by five \( \alpha \)-helices. The AMP-PNP\textsubscript{H} is found bound in the interface between the middle of the \( \beta \)-sheet and several of the \( \alpha \)-helices. The second domain (residues 221–392, rose in Fig. 10) is composed of a four-strand mixed \( \beta \)-sheet and four \( \alpha \)-helices. The majority of protein-ATP contacts (green in Fig. 10) originate in domain 1, although there are two that come from domain 2. The residues studied in this report have been mapped to the GyrB structure (Fig. 10). Gly\textsuperscript{110} (Gly\textsuperscript{114} in GyrB) is in blue and is known to contact the ATP (9). However, neither Ser\textsuperscript{123} (Ser\textsuperscript{127} in GyrB), colored red, nor T201A (Arg\textsuperscript{204} in GyrB), colored magenta, are near the ATP. The closest that any atom of either of these two residues comes to the ATP is about 16 \( \AA \) and, in general, the distance is 20 \( \AA \) or greater. Thus, it is very unlikely that they are involved directly in either ATP binding or the chemistry of ATP hydrolysis.

Based on electron micrographs of DNA gyrase (20, 21), and the crystal structures of the N-terminal fragment of GyrB (9) and the central domain (residues 420–1178) of yeast topoisomerase II (22), it has been proposed that for the type II topoisomerases, effectively, GyrB sits atop GyrA (22). Thus, communication between the ATP-binding and DNA cleavage domains is likely to pass back and forth through the N-terminal domain of ParE, to its C-terminal domain, to the N-terminal domain of ParC. In the GyrB structure, Ser\textsuperscript{127} is at the base of the \( \alpha \)-helix composed of residues 118–126, connecting it to the middle of the first four strands of the six-stranded anti-parallel \( \beta \)-sheet in domain 1. This \( \alpha \)-helix contains Gly\textsuperscript{115}, one of the residues that contacts the ATP. Thus, it is possible that in Topo IV, Ser\textsuperscript{125} could be involved in the propagation of a conformational change elicited by either the binding or hydrolysis of ATP. A crucial role such as this is consistent with the fact that Ser\textsuperscript{125} is conserved in all type II topoisomerases except the one from Mycobacterium leprae (14).

It is more difficult to suggest a role for Thr\textsuperscript{201} based on the known structure. This residue is not conserved at all (14). In GyrB, Arg\textsuperscript{204} is a surface residue, completely solvent exposed. This residue is on the strand of the two-stranded parallel \( \beta \)-sheet that connects it to the six-stranded anti-parallel \( \beta \)-sheet in domain 1. This strand is connected directly to the \( \alpha \)-helix network that provides most of the ATP contacts. Our working hypothesis, therefore, is that altering this residue also disrupts propagation of a conformational change. It would be very interesting to see if a R204A mutation in GyrB has the same biochemical phenotype as ParE T201A.

ATP hydrolysis and binding of DNA to the DNA gate are clearly related, because the latter stimulates the former significantly. ATP is likely to bind separately to the two ATP-binding sites (23) and hydrolysis of one ATP and release of the products occur before the second ATP is hydrolyzed (24). Based on an analysis of the activities of DNA cleavage-defective mutant yeast topoisomerase II proteins, Morris et al. (25) have proposed that a DNA cleavage-dependent conformational change occurs after ATP binding but before hydrolysis. Lindsey and Wang (26) demonstrated, using a mixed heteromer of wild-type and ATPase-defective yeast topoisomerase II protomers, that ATP binding to only one of the subunits was sufficient to elicit a measurable conformational change in the enzyme.

In our case, hyper-DNA cleavage is observed in the absence of ATP and is essentially unaffected by the presence of either ATP or AMP-PNP\textsubscript{H}. This defect can be associated with a decrease in the rate of resealing of the cleaved DNA. This suggests that after the enzyme opens the DNA gate, a signal must be transmitted from the ATP domain to the cleavage domain that is dependent on ATP hydrolysis and not ATP binding (because one of the mutant proteins binds ATP) in order for resealing of the DNA to occur. Thus, it is possible that the order of events might be that DNA bound to the G gate stimulates ATP binding. This closes the N gate, which triggers DNA cleavage and opening of the G gate. Hydrolysis of one ATP then signals that the T segment has exited the ATP-dependent clamp and passed through the G gate, allowing it to close. Hydrolysis of the second ATP might only occur after the T segment has been released from the enzyme through the C gate, allowing the N gate to re-open. Of course, other pathways of coupling ATP hydrolysis to DNA cleavage and strand passage are possible.

In the case of the ParE G110 and ParE S123L mutant enzymes, the observed spontaneous cleavage might arise simply as a result of an increased probability, as the G gate remains open for a longer than normal period, that the scissile phosphate will be transferred to water. The more extreme effect observed with the ParE T201A enzyme suggests that disruption of the normal flow of information via conformational change can also destabilize the enzyme completely, perhaps because the C gate opens before the G gate has closed.

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