Mutational Analysis of *Escherichia coli* Topoisomerase IV

III. IDENTIFICATION OF A REGION OF ParE INVOLVED IN COVALENT CATALYSIS*

(Received for publication, August 12, 1999, and in revised form, November 17, 1999)

Soon Bahng‡, Elena Mossessova§, Pearl Nurse‡, and Kenneth J. Marians‡§

From the ‡Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, and §Molecular Biology Graduate Program, Cornell University Graduate School of Medical Sciences, New York, New York 10021

The products of three dominant-negative alleles of *parE*, encoding the ATP-binding subunit of topoisomerase IV (Topo IV), were purified and their activities characterized when reconstituted with ParC to form Topo IV. The ability of the ParE E418K, ParE G419D, and ParE G442D mutant Topo IVs to bind DNA, hydrolyze ATP, and close their ATP-dependent clamp was relatively unaffected. However, their ability to relax negatively supercoiled DNA was compromised significantly. This could be attributed to severe defects in covalent complex formation between ParC and DNA. Thus, these residues, which are far from the active site Tyr of ParC, contribute to covalent catalysis. This indicates that a dramatic conformational rearrangement of the protein likely occurs subsequent to the binding of the G segment at the DNA gate and prior to its opening.

Type II topoisomerases are capable of passing segments of duplex DNA through transient double-strand breaks in a reaction that is coupled to ATP-binding and hydrolysis. When the passed segment (the T segment) and the transient double-strand break (the DNA gate) are on the same circular DNA molecule, a change in the linking number of the DNA results. When the two segments are on two different DNA molecules, catenation or decatenation occurs. In prokaryotes, the ATP binding and hydrolysis and the DNA cleavage and religation activities reside on different subunits that associate to form a heterotetramer (1–3).

Even though ParC, the DNA cleavage and religation subunit of *Escherichia coli* topoisomerase IV (Topo IV)

1

is a stable dimer in solution (4), it cannot cleave DNA by itself, it must be associated with the ATP-binding subunit, ParE (4, 5). Because ParC binds DNA nearly as well as the intact enzyme, 2

this implies that ParE either contributes amino acid residues to the active site that are necessary for catalysis or that signals from ParE, in the form of conformational changes presumably driven by either the binding or hydrolysis of ATP, are required for DNA cleavage to proceed. As described in an accompanying article (6), Topo IV proteins reconstituted from ParC and mutant ParE proteins that are defective in either ATP binding or hydrolysis or are still capable of covalent complex formation and DNA cleavage. This suggests that there must be other amino acid residues of ParE that are not involved in either ATP binding or hydrolysis, but are required for covalent catalysis. We have identified several such residues in this report.

We isolated six independent alleles in a screen for dominant-negative mutations in *parC* (7). The corresponding mutant ParE proteins all formed catalytically inactive Topo IV when reconstituted with ParC. In an accompanying article (6), we describe the characterization of three of the mutant Topo IV proteins that had defects in ATP binding and hydrolysis. Here, we report our characterization of the ParE E418K, ParE G419D, and ParE G442D mutant Topo IV proteins. While these proteins were relatively unaffected in their ATP-directed functions, they were severely reduced in their ability to form covalent complex or cleave DNA: a reaction mediated by ParC. Given the current understanding of the structure of type II topoisomerases (8), this indicates that a large conformational rearrangement is required to bring these amino acid residues in ParE in the vicinity of the active site Tyr in ParC in order to open the DNA gate.

MATERIALS AND METHODS

Reagents, Enzymes, and DNAs—Reagents, enzymes, and DNAs are all as described in the accompanying articles (6, 7).

Topo IV Binding to DNA—Binding to linear pBR322 DNA was as described by Peng and Marians (9). *K*  values were calculated using the Hill equation as described (10). Binding to a duplex 24-nucleotide-long oligonucleotide composed of a defined Topo IV binding site was as described by Marians and Hiasa (11).

DNA Cleavage Assay—DNA Cleavage Assay was as described by Peng and Marians (12).

Other Assays—Assays for ATP hydrolysis, ParE dimer formation, decatenation of *C. trichodiaphora* kinetoplast DNA (kDNA), ParC covalent complex formation, and fast protein liquid chromatography gel filtration of Topo IV were as described in the accompanying articles (6, 7).

RESULTS

The Mutant Topo IVs Can Hydrolyze ATP and Close Their ATP-dependent Clamps—ParE is the ATP binding and hydrolysis subunit of Topo IV (4, 5). In the first article in this series, we described six mutant ParE proteins that, when reconstituted with ParC, gave Topo IV proteins that were unable to relax negatively supercoiled DNA (7). Three of these, the ParE G110S, ParE S123L, and ParE T201A Topo IV, proteins also exhibited hyper-DNA cleavage. These enzymes were shown to be defective in ATP hydrolysis and DNA religation (6). Here, we investigate the underlying defects in the other three mutant Topo IVs.

All three of the mutations arose in amino acid residues that are highly conserved between type II topoisomerases. Glu 218

and Gly 219 are part of the EGDSA motif that is conserved in all type II topoisomerases (12). Gly 242 is part of the PLRGKILN motif and is conserved in all type II topoisomerases except *Caenorhabditis elegans* 2C, where the corresponding residue is an Arg (12). None of these residues are present in the structure of AMP-P(NH)P bound to the N-terminal fragment of GyR (13). Thus, it is unlikely that they are involved in either ATP binding or hydrolysis.
binding or hydrolysis directly. However, these amino acid substitutions could still affect these activities; Ser^{127} and Thr^{201} are also distant from the ATP, but they affect binding and hydrolysis (7).

The ability of the ParE E418K, ParE G419D, and ParE G442D Topo IV proteins to hydrolyze ATP was therefore examined (Fig. 1). ATP hydrolysis by the ParE E418K and ParE G442D enzymes was essentially indistinguishable from that of the wild type protein, whereas the specific activity of the ParE G419D Topo IV was about one-third that of the wild type. This was a modest decrease in activity, particularly when compared with the defect in ATPase activity manifested by the ParE G110S, ParE S123L, and ParE T201A Topo IVs, where activity was decreased to between one-fifteenth and one-fortieth that of the wild type (7).

Because the ParE E418K, ParE G419D, and ParE G442D Topo IVs could all hydrolyze ATP, they should also be able to dimerize in the presence of AMP-P(NH)P. This is measured by protein-protein cross-linking in the presence of dimethyl suberimidate. All three of the mutant ParE proteins could be cross-linked to give a dimer of ParE in the presence of AMP-P(NH)P (Fig. 2). Thus, none of these three mutants were defective in either of the signature activities of ParE: ATP hydrolysis or the ability to close their ATP-dependent clamp.

Although neither the ParE E418K, ParE G419D, nor ParE G442D Topo IVs could relax DNA, at very high concentrations the ParE G442D enzyme appeared to be able to catenate DNA (7). We therefore examined the ability of the mutant enzymes to decatenate kDNA (Fig. 3). At 40 μM ATP, a concentration sufficient to support maximal rates of decatenation by the wild type enzyme (Fig. 3A), none of the mutant proteins could decatenate DNA when present at equivalent concentrations as the wild type (Fig. 3, B–D). At both higher protein and ATP concentrations, however, the ParE G442D Topo IV could decatenate the kDNA (Fig. 3E). A roughly 100-fold greater concentration of the mutant protein than the wild type effected about the same level of decatenation. As argued in the accompanying article (6), it is unlikely that this activity is a result of wild type ParE contaminating the preparation of ParE G442D.

The Mutant Topo IVs Are Defective in Covalent Catalysis—The mutant Topo IVs were not significantly defective in ATP hydrolysis and could close their ATP-dependent clamp. This suggested that they were defective either in binding DNA or in covalent catalysis. A significant defect in DNA binding seemed unlikely, because the ATPase activity of all the mutant proteins was stimulated by DNA. This was checked directly by measuring the ability of the mutant Topo IVs to bind to linear pBR322 DNA using a nitrocellulose filter binding assay (Fig. 4). The ParE E418K Topo IV bound DNA as well as the wild type, with K_D values of 16.5 and 12.5 nM, respectively. Both the ParE G419D and ParE G442D Topo IVs showed modest defects in DNA binding, giving K_D values of 51.2 and 66.3 nM, respectively. This is unlikely to account for their inability to relax negatively supercoiled DNA, because no activity was manifest even at concentrations of protein as high as 1 μM.

The Mutant Topo IVs Can Close Their ATP-Dependent Clamps—Dimethyl suberimidate cross-linking (23 °C for 30 min) of either the wild type (WT) or mutant ParE proteins (all at 2 μM) in either the presence or absence of AMP-P(NH)P as indicated was as described under “Materials and Methods.” Analysis was by 10% SDS-PAGE. The relative mobility of the cross-linked ParE dimer is discussed in an accompanying article (6).

In general, the cleavage-religation equilibrium is far to the side of religation. This acts to protect the cell from the acciden-

![Fig. 1. The mutant Topo IVs can hydrolyze ATP.](Image)

![Fig. 2. The mutant Topo IVs can close their ATP-dependent clamps.](Image)
tal generation of a lethal double-strand break. Thus, it was possible that the lack of cleavage observed for the mutant Topo IV proteins derived from a shift in the cleavage-religation equilibrium even further toward religation. The quinolone family of antibacterials affect bacterial type II topoisomerase activity by shifting the cleavage-religation equilibrium toward cleavage. Thus, they would be expected to increase DNA cleavage in the assay shown in Fig. 5. That experiment was performed in the absence of such drugs.

We have developed a very sensitive assay for formation of the Topo IV-DNA-quinolone ternary complex. It makes use of the fact that Topo IV cannot bind in a stable fashion to a duplex 24-nucleotide-long oligonucleotide composed of a defined Topo IV binding site sequence in the absence of quinolone (11). Binding is measured using nitrocellulose filters. The ability of the wild type and mutant enzymes to bind to the 24-mer in the presence of 0.5 mM norfloxacin was therefore determined (Fig. 6). Only binding by the wild type enzyme could be detected; no binding for any of the mutant Topo IVs was evident. This suggested that the observed DNA cleavage defect derived from an inability to form the covalent complex. This was tested directly.

If Topo IV is allowed to come to equilibrium bound to uni-
formally 32P-labeled DNA followed by digesting the DNA with nuclease, a fraction of the label will be transferred to the ParC subunit as a result of the Topo IV-DNA complex becoming destabilized and falling apart. Analysis by SDS-PAGE can be used to reveal the label transfer. When treated in this fashion, covalent complex formation by the mutant Topo IVs could not be detected, although it was clearly evident with the wild type enzyme (Fig. 7).

ParE G419D Cannot Form a Stable Heterotetramer with ParC—As discussed above, even though ParC can bind DNA, it cannot form a covalent complex with DNA in the absence of heterotetramer formation with ParE. Thus, the failure of the mutant Topo IVs to form a covalent complex could be because of the lack of a stable association between the mutant ParE proteins and ParC. This was examined by fast protein liquid chromatography gel filtration chromatography. Both ParE E418K and ParE G442D formed heterotetramers with ParC that could be isolated by gel filtration (data not shown). On the other hand, the combination of ParE G419D and ParC clearly did not elute during gel filtration as a heterotetramer (Fig. 8). However, ParE G419D did elute slightly ahead of the position of free ParE. This suggested that it did interact with ParC, but that the interaction was weakened compared with the wild type.

**DISCUSSION**

Type II topoisomerases catalyze the formation and resealing of transient double-strand DNA breaks. During cleavage, the 5′-phosphates of the DNA break are transferred to the enzyme via the formation of a phosphotyrosine bond. Religation reverses this reaction. Cleavage of the DNA corresponds to opening of the DNA gate on the enzyme through which another segment of DNA (the T segment) is passed to the interior of the protein. Once the DNA gate is closed by religation of the break, the topology of the DNA substrate has been altered.

Opening of the DNA gate clearly requires a large conformational rearrangement of the enzyme. Two crystal structures give us an idea of the movement required. The structure of the central core of the yeast topoisomerase II (14) presumably represents the open conformation. Here, the dimer-related active site tyrosines are over 27 Å apart, and it was estimated that they would have to move 35–40 Å toward and past each other to assume an appropriately staggered position to catalyze DNA cleavage. The structure of a 59-kDa N-terminal fragment of GyrA (equivalent to the A9 region of the yeast topoisomerase II structure) presumably represents the closed conformation (15). Here the active site tyrosines are appropriately staggered, but the inter-tyrosyl distance (30 Å) is larger than the width of a double helix of DNA, suggesting that some additional rearrangement of either the DNA or the enzyme must occur to bring the tyrosines close enough to the scissile bonds to effect cleavage.

The chemical mechanism of DNA cleavage and religation remains obscure. Based on the GyrA59 structure, Cabral et al. (15) proposed that the active site of the breakage-reunion activity is formed from Tyr122, which forms the covalent bond with DNA, and Arg121 from one monomer and His80, Arg32, and Lys32 from the other monomer. Berger et al. (16) noted a structural similarity between domain 1 of bacterial topoisomerase I and the B′ region of the yeast topoisomerase II. In the type I enzyme, this domain lies very close to the active site Tyr (17). Both domains contain a nearly invariant set of acidic residues,
Glu9, Asp111, Asp113, and Glu115 in the bacterial enzyme and Glu449, Asp526, Asp528, and Asp530 in the yeast enzyme. Glu449 is part of the conserved EGDSA motif (12). These residues are also part of the toprim motif identified by Aravind et al. (18) on the basis of amino acid sequence comparisons. Toprim is a proposed conserved catalytic domain in type 1A and type II topoisomerases, DnaG-type primases, OLD family nucleases, and RecR proteins. It has been proposed that these acidic residues coordinate Mg$^{2+}$ in bacterial topoisomerase I (17). In the existing structure of the yeast enzyme (14), these residues project away from the active-site Tyr (Fig. 9). Thus, Berger et al. (16) proposed that a rotation of the B’ domain about the linker to the A’ domain to bring these residues in close proximity to the active-site Tyr is effected in the conformation of the enzyme where DNA is bound across the DNA gate and is about to be cleaved. Our finding of amino acid substitutions in the B’ region of ParE that affect covalent complex formation by Topo IV is consistent with this proposal. In our analysis of the biochemical properties of ParE proteins encoded by dominant-negative alleles of parE, we found two groups of mutations (7). When reconstituted with ParC, the ParE G110S, ParE S123L, and ParE T201A Topo IVs were defective in ATP hydrolysis and exhibited hyper-DNA cleavage. The characterization of these proteins is described in the accompanying report (6). The ParE E418K, ParE G419D, and ParE G442D Topo IVs were unaffected in their ability to either hydrolyze ATP or to close their ATP-dependent clamp; however, none of them could relax negatively supercoiled DNA. This inactivity could be attributed to a defect in the ability of these mutant enzymes to form the covalent intermediate.

Although two of these proteins, the ParE G419D and ParE G442D Topo IVs, did show a modest decrease in DNA binding, this was unlikely to account for the defect in covalent complex formation. These experiments were performed at Topo IV concentrations of 60 nM. Thus, even in the case of the ParE G442D Topo IV, which binds linear DNA with a $K_d$ of 66 nM, about 5-fold that of the wild type, 50% of the DNA in the experiment will be bound at any time. Yet, covalent complex formation was undetectable. We therefore conclude that these amino acid substitutions interfere with covalent catalysis.

Glu418 is part of the toprim (18) and EGDSA (12) motifs and was predicted to be directly involved in covalent catalysis by coordinating Mg$^{2+}$ (17). If this is in fact the case, the reversal of charge effected by the E418K substitution would clearly have a profound effect on covalent catalysis. An equivalent substitution, E449A, inactivates the DNA relaxation and covalent complex-forming activities of yeast topoisomerase II (19). Gly419 is also part of the EGDSA motif (12). However, the lack of a functional group makes it unlikely that this residue participates directly in covalent catalysis. It seems likely that the G419D substitution imposes an effect on catalysis by interfering with the hydrogen-bonding network that would be required to coordinate Mg$^{2+}$. In the yeast topoisomerase II structure (14) the equivalent residue, Gly450, lies very close to Glu449 (Glu418 in ParE), within 2–4 Å, is fairly close to Asp526, within 5–7 Å, and is further from Asp528 and Asp530, from 7 to >10 Å (Fig. 9). These latter three Asp residues are part of the putative acidic tetrad necessary for Mg$^{2+}$ coordination. Intrusion of an
additional negative charge into this region could easily shift the pattern of hydrogen bonding between the Mg ion and the acidic tetrad. Consistent with this is that in topoisomerase II from Candida albicans, the Asp in the EGDSA motif is a Leu (12) and that substitution of the Ser with Ala in the yeast topoisomerase II does not inactivate the enzyme (19). Neither of these two amino acid replacements would be expected to interfere with a hydrogen-bonding network.

Gly442 is part of the conserved PLRGKILN motif. In the yeast enzyme, substitutions of the Arg and Lys in this motif with Ala has no effect on the catalytic activities of the topoisomerase (20), whereas Ala substitution of the Asn does ablate the superhelical DNA relaxation activity and reduce covalent complex formation to about one-tenth the level of the wild type (19). This suggests that residues in this motif may not be directly involved in catalysis. Consistent with this is the observation that the ParE G442D Topo IV retained some catalytic activity. This enzyme could decatenate kDNA at concentrations 100-fold greater than that required for the wild type enzyme. Clearly, this implies that this mutant enzyme retains some low level ability to form the covalent complex. Our inability to detect this presumably relates to an inability to approach the protein concentrations that would be required in the assay for covalent complex formation.

Gly442 is considerably farther from the acidic tetrad than Gly419. In the yeast topoisomerase II structure (14), the equivalent residue, Gly476, is 8–9 Å from Glu449 (Glu418 in ParE), 7–9 Å from Asp526 and Asp528, and greater than 12 Å from Asp530. Thus, whereas the G442D substitution could interfere with the hydrogen-bonding network involving the acidic tetrad, it is less likely.

By making mixed heterodimers of yeast topoisomerase II where one protomer carried a mutation in this region of the B' domain and the other protomer carried the Y782F mutation that inactivates covalent complex formation, Liu and Wang (19) have shown that amino acid residues in the B' domain, such as the acidic tetrad, cooperate in trans with the A' region of the other protomer in the dimer. The structural organization of this conformation is unknown. Thus, an explanation for the disruptive effect of the ParE G442D substitution may only become apparent when additional crystal structures are available.

It is clear, however, that our data and that from other laboratories discussed above, point up another of the large conformational changes necessary for activity of the type II topoisomerases. The cooperation in trans between the B' and A' regions of the two halves of the enzyme presumably contributes to the stabilization of the protein as the DNA gate opens. This view is supported by the apparent instability of the ParE G419D Topo IV tetramer.

REFERENCES
1. Wang, J. C. (1996) Annu. Rev. Biochem. 65, 635–692
2. Burden, D. A., and Osheroff, N. (1998) Biochim. Biophys. Acta 1400, 139–154
3. Levine, C., Hiasa, H., and Marians, K. J. (1998) Biochim. Biophys. Acta 1400, 29–43
4. Peng, H., and Marians, K. J. (1999) J. Biol. Chem. 268, 24481–24490
5. Kato, J., Suzuki, H., and Ikeda, H. (1992) J. Biol. Chem. 267, 25676–25684
6. Nurse, P., Bahng, S., Mosessonova, E., and Marians, K. J. (2000) J. Biol. Chem. 275, 1400–1411
7. Mosessonova, E., Levine, C., Peng, H., Nurse, P., Bahng, S., and Marians, K. J. (2000) J. Biol. Chem. 275, 4099–4103
8. Berger, J. M. (1998) Biochim. Biophys. Acta 1400, 3–18
9. Peng, H., and Marians, K. J. (1999) J. Biol. Chem. 270, 25286–25290
10. Yong, Y., and Romano, L. (1995) J. Biol. Chem. 270, 24509–24517
11. Marians, K. J., and Hiasa, H. (1997) J. Biol. Chem. 272, 9401–9409
12. Caron, P. R. (1999) Methods Mol. Biol. 94, 279–316
13. Wigley, D. B., Davies, G. J., Dodson, E. J., Maxwell, A., and Dodson, G. (1991) Nature 351, 624–629
14. Berger, J. M., Gamblin, S. J., Harrison, S. C., and Wang, J. C. (1996) Nature 379, 225–232
15. Cabral, J. H. M., Jackson, A. P., Smith, C. V., Shikotra N., Maxwell, A., and Liddington, R. C. (1997) Nature 388, 903–906
16. Berger, J. M., Fass, D., Wang, J. C., and Harrison, S. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7876–7881
17. Lima, C. D., Wang, J. C., and Mandragon, A. (1994) Nature 370, 138–146
18. Aravind, L., Leipe, D. D., and Koonin, E. V. (1998) Nucleic Acids Res. 26, 4205–4213
19. Liu, Q., and Wang, J. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 881–886
20. Wasserman, R. A., and Wang, J. C. (1994) J. Biol. Chem. 269, 20943–20951