**Escherichia coli** topoisomerase IV (topo IV) is an essential enzyme that unlinks the daughter chromosomes for proper segregation at cell division. *In vitro*, topo IV readily distinguishes between the two possible chiralities of crossing segments in a DNA substrate. The enzyme relaxes positive supercoils and left-handed braids 20 times faster, and with greater processivity, than negative supercoils and right-handed braids. Here, we used chemical cross-linking of topo IV to demonstrate that enzyme bound to positively supercoiled DNA is in a different conformation from that bound to other forms of DNA. Using three different reagents, we observed novel cross-linked species of topo IV when positively supercoiled DNA was in the reaction. We show that the ParE subunits are in close enough proximity to be cross-linked only when the enzyme is bound to positively supercoiled DNA. We suggest that the altered conformation reflects efficient binding by topo IV of the two DNA segments that participate in the strand passage reaction.

The genome of *Escherichia coli* contains 4.6 megabases of DNA in a single circular chromosome that resides in a nucleoid of less than a μm^3^ (1). As a consequence of this tight packing of DNA into a confined space, the chromosome may become tangled during replication or recombination. Removal of these detrimental tangles is accomplished by type I and type II topoisomerases, which transiently cleave one or both strands of DNA, respectively, and pass another strand or duplex through the break before religation (for review, see Ref. 2). *E. coli* has two essential type II enzymes: DNA gyrase and topoisomerase IV (topo IV) (for review, see Ref. 3). Gyrase is required to maintain the negative ([−]) supercoiling of the chromosome by virtue of its unique ability to introduce ([−]) supercoils into DNA. This same activity serves during DNA replication to remove positive ([+]) superhelical stress generated ahead of the fork due to unwinding of the DNA duplex. While topo IV assists gyrase in removing ([+]) supercoils during DNA replication, its critical role is unlinking the intertwined replicated chromosomes to allow segregation of one copy to each daughter cell at division (4–6).

A remarkable feature of topoisomerases that is key to their *in vivo* function is the ability to discriminate among DNA substrates differing in topology. This substrate specificity is intrinsic, as it is exhibited *in vitro* by purified enzymes. For example, topo IV removes catenane and ([+]) supercoil links at least 20 times faster than ([−]) supercoils (7–10). The active form of topo IV is a tetramer composed of two ParC and two ParE subunits (Fig. 1A) (11). During the catalytic cycle, topo IV binds the gate (G) segment of DNA (Fig. 1B). Upon binding of a second DNA segment, the transport (T) segment, the ParE subunits dimerize around the T segment DNA. The enzyme then cleaves the G segment, passes the T segment through the break and reseals the broken duplex. Topo IV DNA binding and cleavage can proceed in the absence of ATP *in vitro*, whereas ATP hydrolysis is required for strand passage (8, 12). The basis for substrate discrimination by topo IV was found to be the relative orientation of the G and T segments (9, 10, 13). The DNA superhelix is left-handed in positively supercoiled (([+])-SC) DNA and right-handed in negatively supercoiled ([−])-SC DNA (Fig. 1C). Topo IV removes left-handed crossings 20 times faster than right-handed crossings (9, 10). Thus, topo IV relaxes ([−])-supercoils more efficiently, because DNA segments with the preferred chirality are found with much higher probability in ([+])-SC DNA (9).

Additionally, topo IV is far more processive on substrates containing left-handed crossings, removing as many as 80 left-handed but only one or two right-handed crossings in a single burst of activity (8–10). This difference in processivity suggests that, after release of a transported T segment, topo IV has a higher probability of capturing a new T segment with ([+])-SC than with ([−])-SC DNA before dissociating from the substrate. There may also be a difference in the mode of binding of topo IV to ([+])-SC DNA that allows it to remain bound between strand passages.

Filter binding assays indicated that topo IV binds equally well to ([+]) and ([−])-SC DNA (9). However, this assay does not distinguish tetramers bound only to a G segment from those bound to both G and T segments. We wished to examine more directly the interaction of topo IV with DNA. In this report, we used chemical cross-linking of topo IV bound to ([−]) and ([+])-SC DNA to explore whether substrate binding altered the conformation of the enzyme. We found that ([+])-SC DNA promoted the formation of a number of cross-linked species not detected in the absence of DNA or in the presence of other...
topological forms of DNA. Interestingly, we observed cross-linking of ParE into dimers only when topo IV was bound to (+)-SC DNA. As ParE dimerization occurs after T segment binding, we interpret the cross-linking of ParE dimers as a reporter for tetramers that have bound both the G and T segments.

EXPERIMENTAL PROCEDURES

Proteins—Topoisomerase IV (7), wheat germ topoisomerase I (14), and the archaeal histone HMfB (15) were purified as described.

DNA Substrates—The DNA used in all experiments was pUC18 (2.7 kb), except for the preparation of knots and catenanes, which used the 7-kb plasmids pAB3 and pAB4, respectively (16). DNAs of different (-) and (+)-SC were prepared as described (8).

Cross-linking Reactions and Analyses—Topo IV cross-linking reactions contained 200 nM topo IV, 100 nM DNA, 25 mM HEPES (pH 7.6), 100 mM potassium acetate, 10 mM MgCl₂, 0.5 mM dithiothreitol, and 5% glycerol in a 10-μl reaction volume. Proteins were incubated with the DNA for 10 min before the addition of the cross-linking reagent. Glutaraldehyde (Poly-science, EM grade) cross-linking was carried out at 30 °C for 10 min and was terminated by the addition of glycine. Sulfo-EGS (ethylene glycol bis(sulfosuccinimidyl succinate)) and BS3 (bis(sulfosuccinimidyl) suberate) (Pierce) cross-linking was at 23 °C for 30 min and was terminated by the addition of Tris, according to the manufacturer’s recommendations. The distance between the reactive groups for the cross-linking reagents is ~7 Å for glutaraldehyde, 11.4 Å for BS³, and 16.1 Å for Sulfo-EGS.

Cross-linked proteins were analyzed on denaturing 4.5% polyacrylamide gels and visualized by silver staining. Western blot analyses used polyclonal mouse antibodies against ParC or ParE. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce). Proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce) and quantified on an Alpha Innotech ChemiImager or Typhoon 9400 PhosphorImager (Amersham Biosciences).

FIGURE 1. Architecture of topo IV and its association with DNA. A, topo IV is a heterotetramer composed of two molecules each of ParC (gray ovals) and ParE (white ovals). B, topo IV binds the G segment and the T segment of DNA. The ParE subunits dimerize prior to strand passage. C, left- and right-handed crossings. We define handedness by the counterclockwise rotation (arrows) of the underlying segment to the overlying segment through the angle θ. For left-handed crossings, as found in the superhelix of (+)-SC DNA, θ < 90°. For the right-handed crossings in (-)-SC DNA, θ > 90°.

TABLE 1

Abundance of topo IV cross-linked species in the absence and presence of ATP

| Species          | % of total protein | −ATP | +ATP |
|------------------|--------------------|------|------|
| Tetramers        | 11                 | 15   |
| Trimers          | 12                 | 17   |
| Dimers           | 24                 | 21   |
| C monomer        | 14                 | 13   |
| E monomer        | 39                 | 34   |

reagents is ~7 Å for glutaraldehyde, 11.4 Å for BS³, and 16.1 Å for Sulfo-EGS.

Cross-linked proteins were analyzed on denaturing 4.5% polyacrylamide gels (80:1 acrylamide:bisacrylamide) in 0.1 M sodium phosphate, 0.1% SDS buffer (pH 7.2) with recirculation of the buffer (17). Proteins were visualized by silver staining or Western blotting. Western blot analyses used polyclonal mouse antibodies against ParC or ParE. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce). Proteins were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce) and quantified on an Alpha Innotech ChemiImager or Typhoon 9400 PhosphorImager (Amersham Biosciences).
Isolation of Topo IV-DNA Complexes—Topo IV cross-linking reactions contained 5 mM Sulfo-EGS and (-)-SC, (+)-SC, or no DNA. The reaction was applied to a sizing column containing a 350-μl bed volume of Sepharose CL-2B resin in 25 mM HEPES (pH 7.6), 0.5 mM dithiothreitol, 0.1 mM EDTA, and 500 mM potassium acetate, the latter to minimize protein aggregation. The fractions containing DNA were determined by analyzing an aliquot of each fraction by agarose gel electrophoresis and Southern blotting. The recovered DNA was linearized by digestion with EcoRI. Protein co-eluting with the DNA was analyzed by Western blotting.

RESULTS

Cross-linking of Topo IV in the Absence of DNA—We first characterized the cross-linking of topo IV without DNA so we could determine whether the presence of DNA caused detectible changes in the enzyme. Topo IV was cross-linked with increasing concentrations of glutaraldehyde (GA), and the products were analyzed by denaturing PAGE (Fig. 2A). We identified dimers, trimers, and tetramers based on their electrophoretic mobility compared with molecular weight standards. We expect that cross-linking between ParC and ParE takes place primarily in the tetramer. Thus, the observed trimers, and some dimers, most likely result from incompletely cross-linked tetramers, which dissociate under the denaturing gel conditions. Importantly, we detected no oligomers larger than tetramers, even at the highest GA concentration, indicating that the cross-linked species reflected subunit interactions within the tetramers and not protein aggregates or dimers of tetramers. When we cross-linked each subunit alone, ParE remained a monomer and ParC was predominantly a dimer (data not shown). For our experiments, we used a relatively low concentration of GA (2 mM), at which monomers, dimers, trimers, and tetramers could be readily detected, enabling us to monitor the effect of DNA on these species (Fig. 2A, lane 7). A low cross-linker concentration also has the advantage of trapping the most stable subunit interactions.

To determine whether the cross-linked oligomers visualized by silver staining contained both ParC and ParE, we utilized Western blotting. Fig. 2B shows the results of probing parallel gels with antibodies specific for ParC (lanes 1 and 2) or ParE (lanes 3 and 4). With cross-linked topo IV, dimers, trimers, and tetramers reacted with both antibodies.
TABLE 2

Molecular mass estimates for cross-linked species

| Species       | Actual molecular mass | Calculated molecular mass ± S.D. |
|---------------|-----------------------|----------------------------------|
| E monomer     | 70                    | 71 ± 5                           |
| C monomer     | 84                    | 86 ± 6                           |
| EE dimer      | 140                   |                                  |
| New band      | 154                   | 141 ± 10                         |
| CE dimer      | 168                   | 164 ± 10                         |

(lanes 2 and 3). The dimer was the major species when ParC was cross-linked alone (lane 1) and the monomer with ParE alone (lane 4).

Cross-linking of Topo IV in the Presence of DNA—Using the characterization of species cross-linked in the absence of DNA as a reference, we investigated whether topo IV was altered when bound to DNA. To prevent relaxation of the supercoiled substrates prior to addition of the cross-linking reagent, we omitted ATP from the reactions. We found that the absence of ATP had no detectable qualitative or quantitative effect on the cross-linked species with topo IV (Table 1) or either subunit alone (data not shown) in the absence of DNA. When we cross-linked topo IV in the presence of (+)-SC DNA, we observed a new dimer species (Fig. 3A, lane 4) that did not form with (−)-SC DNA (lane 3) or with either subunit alone (data not shown). The new band was abundant, comprising 60% of the dimers. Based on its electrophoretic mobility we inferred that the band was a dimer of ParE (EE) (Table 2) and confirmed this assignment by Western blot analysis. With a ParE antibody, there were two dimers with (+)-SC DNA (Fig. 3B, lane 2). The slower migrating band is the CE dimer, based on its larger molecular weight. The faster migrating band must then be the EE dimer, which is present in a 10-fold excess over CE. In contrast, with (−)-SC DNA, greater than 80% of the dimers were the slower migrating CE species (Fig. 3B, lane 3): the small amount of EE dimer was not visible by silver staining (Fig. 3A, lane 3). The ParC antibody detected only one dimer band, which co-migrated with that formed with ParC alone, as in Fig. 2B (data not shown). We expect this is primarily the CC dimer and that the CE dimer was obscured by the abundance of CC.

The EE cross-linking must have occurred in a complex with ParC associated with the (+)-SC DNA, as both subunits and the DNA were required. The appearance of the EE dimer is significant, because it shows that the ParE subunits are close enough to be cross-linked by GA in tetramers bound to (+)-SC DNA, even in the absence of ATP.

Topo IV is the major enzyme in E. coli that unlink DNA knots and catenanes (18, 19). To examine whether these substrates affected topo IV cross-linked species we generated knotted and catenated DNAs using phage λ integrase and cross-linked topo IV in their presence. Fig. 3C shows substantial rearrangement within the topo IV tetramer conformation, we cross-linked topo IV with Sulfo-EGS in the presence of DNAs of different superhelical densities (σ) (Fig. 3E). The cross-linked species with (−)-SC (lanes 2–4), relaxed (lane 5), or linear DNA (lane 9) were the same. In contrast, multiple dimer and trimer bands were visible with all three (+)-SC DNAs tested (lanes 6–8), even when the 2.7-kb plasmid contained only two or three supercoils (σ = +0.01). Thus, only a few left-handed crossings in the DNA are sufficient to promote the alterations in topo IV evidenced by the new cross-linked bands. This experiment also demonstrated that the conformational changes we observed are activated by (+)-SC DNA rather than inhibited by (−)-SC DNA.

Taken together, the results with three cross-linking reagents show that not only are the ParE subunits in closer proximity but also that there is extensive rearrangement within the topo IV tetramers bound to (+)-SC DNA.

Quantification of Topo IV Bound to (−)- and (+)-Supercoiled DNA—As (−)-SC DNA is a poor substrate for topo IV, we wanted to be sure that we were comparing tetramers assembled on (−)- and (+)-SC DNA rather than unbound and bound protein. To address this question, we carried out an experiment to quantify topo IV bound to the DNA after cross-linking. To measure total bound protein, we cross-linked to completion, such that the tetramers would not dissociate on the denaturing gels. We used a high concentration of Sulfo-EGS, at which scant ParC monomer remained. The reaction was then passed over a sizing column, and the fractions containing DNA were collected. The recovered DNA was linearized to allow any proteins topologically linked around the DNA to slide off, and the amount of protein released from the DNA was determined by Western blotting.
Fig. 4 shows the results for reactions containing (−)-SC (lanes 1–7) or (+)-SC DNA (lanes 8–14). Compared with the protein applied to the column (lanes 1 and 8), the protein released from the DNA was highly enriched for tetramers (lanes 4, 5, 11, and 12). When we quantified the amount of tetramer bound to (−)- and (+)-SC DNA in two experiments, we found only about 20% more bound to (+)-SC DNA. The control lanes (16–20) show that no protein eluted in the fractions examined if DNA was omitted from the cross-linking reaction, confirming that we were measuring only DNA-bound tetramers. Thus, even though topo IV binds nearly as well to (−)-SC as to (+)-SC DNA, in agreement with previous filter binding data, most of the protein is in a different conformation when bound to (+)-SC DNA. It is the geometry of (+)-SC DNA that effects the conformational change in topo IV.

**DISCUSSION**

We carried out chemical cross-linking of topo IV to investigate whether enzyme conformation was affected by DNA substrate structure. We demonstrate that tetramers assembled on (+)-SC DNA have a different conformation, as evidenced by the appearance of new cross-linked species when topo IV is treated with three different reagents in the presence of (+)-SC DNA but not linear, relaxed circular, (−)-SC, knotted, or catenated DNA (Fig. 3). We interpret these new cross-linked species as reflecting conformations of topo IV bound to both G and T segments on (+)-SC DNA.

With GA cross-linking, we found that the ParE subunits were in close enough proximity to allow efficient cross-linking only in tetramers bound to (+)-SC DNA. We interpret this juxtaposition of the ParE subunits as the closing of the ParE “gate” that accompanies binding of the T segment (Fig. 5). When topo IV is bound only to the G segment, ParE subunits can be cross-linked to ParC but rarely to each other, as is the case with (−)-SC DNA (Fig. 5A). Here, topo IV cannot readily bind a T segment with the necessary chirality, and most of the ParE-containing dimers released from tetramers upon denaturation also contain ParC (CE dimers). With (+)-SC DNA, topo IV quickly binds a T segment, and the accompanying conformational change enables efficient cross-linking of the ParE subunits to each other (Fig. 5B). Thus, the EE dimer provides an assay to distinguish topo IV bound to G and T segments from those bound to G alone. However, closing of the ParE gate is not the only change accompanying T segment binding. The appearance of multiple species of dimers, trimers, and tetramers when topo IV was cross-linked with Sulfo-EGS and BS3 indicates a global change in the enzyme when bound to (+)-SC DNA.

We observed only a trace of ParE dimer cross-linking with catenated DNA, even though decatenation of daughter chromosomes is a major function of topo IV in vivo. Replication catenanes contain predominantly right-handed DNA crossings, although simulations of catenane conformations showed that left-handed crossings are often found within the loosely catenated substrates encountered in the cell (9). Similarly, left-handed crossings must be found in any DNA that is a substrate for topo IV, including (−)-SC DNA and the integrase catenanes used here. However, the density of such crossings is not high enough in these substrates to promote the efficient T segment binding and ParE dimer cross-linking observed with (+)-SC DNA.

Cross-linking traps the topo IV subunit interactions depicted in Fig. 5. In the absence of cross-linking, the assembly and disassembly of tetramers, as well as their association with and dissociation from DNA, are very likely dynamic processes. At the
beginning of each catalytic cycle, the enzyme is bound only to a G segment (Fig. 5A). This may be an unstable state that is sensitive to tetramer disassembly and/or dissociation from the DNA. If the rate of dissociation is faster than the rate of sequential T segment binding, the tetramer would need to reassemble or rebinding between catalytic cycles. This could be the basis for the observed distributive relaxation of (−)-supercoils. Binding of a T segment and the accompanying conformational change could stabilize the protein and rescue a conformation that is vulnerable to dissociation. With (+)-SC DNA, another T segment can easily be bound, and the tetramer remains on the DNA between strand passages, resulting in high processivity. Thus, the intrinsic chirality preference of topo IV underlies the high processivity of the enzyme on (+)-SC DNA.

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