Flexibility at Gly-194 Is Required for DNA Cleavage and Relaxation Activity of Escherichia coli DNA Topoisomerase I*

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DNA topoisomerases are ubiquitous enzymes involved in DNA replication, transcription, and recombination (reviewed in Refs. 1–3). These enzymes catalyze the interconversion of DNA topological isomers by first cleaving one or more DNA strands and then allowing another single- or double-stranded DNA strand to pass through the cleaved DNA. After reigation of the cleaved DNA, the DNA involved in strand passage has to be released from the enzyme molecule. There are therefore multiple steps in catalysis that would require changes in enzyme conformations. Type IA DNA topoisomerases catalyze the topological conversions by breaking and rejoining a single strand of DNA with the cleaved DNA forming a 5'-phosphoryl-tyrosine linkage with the enzyme. The current models of catalysis for this class of DNA topoisomerases (4–6) begin with the binding of the enzyme at the junction of single- and double-stranded DNA, followed by cleavage of a single G strand of DNA by the active-site tyrosine to create a “gate” for subsequent strand passage. However, the crystal structures of the enzymes in the absence of DNA (7–9) show insufficient space around the active-site tyrosine responsible for G strand DNA cleavage to accommodate the placement of the G strand of DNA in the vicinity of the tyrosine hydroxyl nucleophile. A more recent crystal structure of Escherichia coli DNA topoisomerase III with an 8-base-long single-stranded oligonucleotide bound at the active site showed that an α helix making contacts with the G strand DNA has shifted relative to its position in the structure of E. coli DNA topoisomerase III with no DNA bound (10). The corresponding α helix in E. coli DNA topoisomerase I begins at position 195 (Fig. 1). The glycine residue preceding the N terminus of this α helix (Gly-194) is strictly conserved among type IA DNA topoisomerases (11). The side chains of residues equivalent to Arg-195 and Gln-197 of E. coli DNA topoisomerase I make contacts with the phosphate backbone in the topoisomerase III oligonucleotide crystal structure (10). We hypothesized that the conformational flexibility around the Gly-194 of E. coli DNA topoisomerase I may be important for the movement of this α helix within the enzyme during catalysis so that the active-site tyrosine will be in a position to carry out nucleophilic attack on the scissile phosphate. This was investigated by site-directed mutagenesis of Gly-194 and analysis of the purified mutant enzymes. The results demonstrate that the conformational flexibility around Gly-194 is critical for DNA cleavage to take place to lead to subsequent relaxation of negatively supercoiled DNA.

EXPERIMENTAL PROCEDURES

Enzymes—E. coli DNA topoisomerase I and its mutant derivatives were expressed and purified to >95% homogeneity as described previously (12). Single amino acid substitutions in the enzyme sequence were achieved using the QuickChange mutagenesis kit with Pfu DNA polymerase I (Stratagene) using plasmid pRV10 (13) as a template. The sequence of the top-strand oligonucleotides (custom synthesized by Sigma-Genosys) used in the random replacement of Gly-194 was 5’-GGGCTGTCTCCTGCCCACGTGTGCAGTCG-3’ (N = C + G + A). This sequence did not allow selection for early termination mutations at position 194 found to be favored in other random mutagenesis experiments of E. coli DNA topoisomerase I1 but also excluded the replacement of Gly-194 by phenylalanine, cysteine, and tyrosine.

Relaxation Activity Assay—Wild-type and mutant topoisomerases were diluted serially for a relaxation activity assay in a reaction volume of 20 μl with 10 μM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, 6 mM MgCl₂, and 0.5 μg of CsCl gradient-purified supercoiled plasmid DNA. For the relaxation assay of activity in total E. coli soluble lysates, 10 μg of yeast tRNA was also included to inhibit the nuclease activities in the lysates. The reaction was terminated after a 30-min incubation at 37°C by the addition of 5 μl of 50 mM EDTA, 50% glycerol, and 0.5% (v/v) bromphenol blue. The DNA was analyzed by electrophoresis in a 0.8% (w/v) agarose gel with TAE buffer (40 mM Tris acetate, pH 8.1, 2 mM EDTA). After staining with ethidium bromide, the gel was photographed under UV light.

Cleavage of Single-stranded DNA—To prepare for a cleavage substrate with a strong topoisomerase I cleavage site (14, 15), PCR was carried out with the forward primer (5’-CTCTGGCGGTGATAATG-3’) and reverse primer (5’-CTCTGCCGCTGCCVNNCGTGTGCAGTCG-3’) using phase α DNA as the template and Taq DNA polymerase. The forward primer was labeled prior to the PCR reaction with T4 polynucleotide kinase and [α-32P]ATP so that the top strand of the 203-bp PCR product was labeled at the 5’-end. After electrophoresis in a 14% agarose gel, the PCR product was gel-purified using the GenElute spin column (Sigma). The eluted DNA was ethanol-precipitated and resuspended in 10 μM Tris-HCl, pH 8.0, 1 mM EDTA. Prior to the reaction with topoisomerase, the DNA was denatured to single strands by heating at 95°C for 5 min. After incubation with the wild-type or mutant topoi-
somerase at 37 °C for 30 min, trapping of the covalent enzyme-DNA complex and cleaved DNA was achieved by the addition of 0.1 M NaOH. After neutralization (14), the DNA was electrophoresed in an 8% sequencing gel followed by autoradiography of the dried gel to visualize the 5’-end-labeled DNA cleavage products.

**Gel Electrophoretic Mobility Shift Assay**—A 39-base oligonucleotide 5’-GTATGCAATGGGCTTGGCCAAACGACGAGGATAC3’ (PAGE purified from Sigma-Genosys) with a strong cleavage site for E. coli DNA topoisomerase I (14, 15) was labeled at the 5’-end with T4 polynucleotide kinase and [γ-32P]ATP. Reaction mixtures (10 μl) containing 20 mM Tris-HCl, pH 7.5, 0.1 mg/ml bovine serum albumin, 12% glycerol, 1 pmol of the labeled 39-mer, and 0.5–4 pmol (48–388 ng) of wild-type or mutant topoisomerase I were incubated at 37 °C for 5 min. The formation of the gel electrophoretic mobility shift complex was measured by electrophoresis in a 6% polyacrylamide gel with an electrophoresis buffer of 45 mM Tris borate, pH 8.3, 1 mM EDTA as described (16) and quantitated with the PhosphorImager Storm 760.

**Oligonucleotide Cleavage Assay**—Wild-type and mutant enzymes were incubated with the 5’-end-labeled oligonucleotide used in the gel shift assay (5 pmol in 50 μl) at 37 °C for 5 min, the reaction products were removed and mixed with an equal volume of stop solution (79% formamide, 0.2 M NaOH, 0.04% bromphenol blue). The samples were heated at 80 °C for 5 min before electrophoresis in a 15% sequencing gel. The fraction of oligonucleotide cleaved by the enzyme (following CAAT↓ in the oligonucleotide sequence) was determined by quantitative analysis with the PhosphorImager Storm 760.

**Nuclease P1 and DNase I Digestion**—Conditions were modified from those described previously (17). For nuclease P1 digestion, the 5’-end-labeled 39-base oligonucleotide (0.2 pmol) was incubated with increasing amounts of wild-type and mutant topoisomerase I in 5 μl of 40 mM Tris-HCl, pH 8.0, 0.1 mg/ml bovine serum albumin, 1 mM magnesium chloride for 4 min at 37 °C. Nuclease P1 (100 ng from Roche Applied Science) was added, and incubation was continued for 2 min before the addition of EDTA to 10 mM and an equal volume of 90% formamide, 0.2 M NaOH, 0.04% bromphenol blue. After heating at 95 °C for 5 min, the reaction products were electrophoresed in a 15% sequencing gel and visualized with the PhosphorImager Storm 760. The same procedures were used for DNase I digestion except that 0.2 pmol of a 59-base-long oligonucleotide with the same preferred topoisomerase I cleavage site near the junction of the single- and self-complementary double-stranded regions (5’-GCCC-TGAAAAGATTATGGCAAT↓GCCCTTTGGGCAAAACGACGAGGATAC3’ labeled at the 5’-end) was used as the substrate with 50 ng of DNase I added to each reaction.

**Limited Proteolytic Digestion**—Limited digestion of wild-type and mutant topoisomerase I enzymes was carried out with 5 μg of protein in 20 mM potassium phosphate, pH 7.5, 20 mM KCl, 0.2 mM dithiothreitol using sequencing grade Glu-C endoproteinases (Roche Applied Science) at 1:100 w/w ratio at 37 °C for the indicated lengths of time. The digestion was stopped by the addition of an equal volume of 2× gel loading buffer for SDS gel and immersion in boiling water for 5 min. The digestion mixtures were analyzed by electrophoresis in a 15% SDS-polyacrylamide gel followed by staining with Coomassie Blue.

**Intrinsic Tryptophan Fluorescence Measurements**—Fluorescence measurements were performed with the CARY Eclipse fluorescence spectrophotometer with excitation and emission (25°C). The spectral bandwidths were 5 and 10 nm, respectively, for excitation and emission. The wild-type and mutant topoisomerase I were present at 0.1 mg/ml in 20 mM potassium phosphate, pH 7.4, 20 mM KCl.

**RESULTS**

**Substitution at Gly-194 in the Potential Hinge Region Resulted in Loss of Relaxation and DNA Cleavage Activities**—Random substitutions were generated for Gly-194 by oligonu-
cleotide-directed in vitro mutagenesis, and six different substitution mutants for Gly-194 were identified by DNA sequencing of the individual plasmid pRV10 DNA isolates (Fig. 2). Western blot analysis of the expression level of the mutated topoisomerase in the total protein lysates of *E. coli* GP200 (*H9004* topA) cells expressing these mutant topoisomerases showed that all six mutant topoisomerases were accumulated in the soluble extracts at levels comparable with wild-type topoisomerase I (Fig. 2a), so the mutations at Gly-194 did not appear to have resulted in insoluble or unstable topoisomerase I proteins. When assayed with negatively supercoiled plasmid DNA, no relaxation activity was detectable in the total lysates of *E. coli* GP200 (*H9004* topA) cells expressing these six mutant topoisomerases (Fig. 2b). Relaxation activity was readily detectable for the GP200 lysate expressing the wild-type topoisomerase I even after a 10-fold dilution of the lysate. The G194R and G194A mutant enzymes expressed in GP200 were purified to >95% homogeneity with procedures similar to those used for the wild-type topoisomerase I and assayed for relaxation activity (Fig. 3). The G194R mutant topoisomerase I had no detectable relaxation activity, whereas the G194A mutant enzyme had an ~40-fold reduction in activity when compared with the wild-type topoisomerase I.

The DNA cleavage activity of the purified mutant topoisomerases was assayed first with a 203-base long single-stranded DNA generated from *λ* DNA by PCR. This single-stranded DNA has a preferred topoisomerase I cleavage site identified previously (14, 15). Interaction with up to 480 ng of the G194R and G194A mutants did not yield any detectable cleavage product (Fig. 4). The cleavage product from 7.5 ng of wild-type topoisomerase I was visible, so the cleavage activity of the mutants was reduced ~50-fold.

The rate of DNA cleavage activity was also assayed with a 39-base-long oligonucleotide containing the same strong topoisomerase I cleavage site (5 pmol) was incubated with different amounts of wild-type or G194A mutant enzyme, and the time course of cleavage was followed by gel electrophoresis and PhosphorImager analysis.

*The Non-covalent Interaction with DNA Is Only Moderately Decreased by the Gly-194 Mutations*—The non-covalent binding of wild-type and mutant topoisomerases to the 5’-end-labeled 39-base oligonucleotide substrate was compared using the gel mobility shift assay (Fig. 6). Up to 4 pmol of enzyme was added to the oligonucleotide. A higher amount of enzyme was not used to limit the formation of the covalent cleavage product from the wild-type topoisomerase I. The G194R mutant topoisomerase I and assayed for relaxation activity (Fig. 3). The G194R mutant topoisomerase I had no detectable relaxation activity, whereas the G194A mutant enzyme had an ~40-fold reduction in activity when compared with the wild-type topoisomerase I.

The amount of non-covalent complex formed by the G194R mutant enzyme was only slightly reduced when compared with the wild-type topoisomerase I. The amount of non-covalent complex formed by the G194R mutant enzyme was only slightly reduced when compared with the wild-type topoisomerase I. The amount of non-covalent complex formed by the G194R mutant enzyme was only slightly reduced when compared with the wild-type topoisomerase I. The amount of non-covalent complex formed by the G194R mutant enzyme was only slightly reduced when compared with the wild-type topoisomerase I. The amount of non-covalent complex formed by the G194R mutant enzyme was only slightly reduced when compared with the wild-type topoisomerase I.
significantly affected by the G194A mutation as the DNA cleavage step.

The non-covalent complexes formed between wild-type and mutant topoisomerase I with the 39-base oligonucleotide were further compared using nuclease P1 digestion. In the presence of Mg$^{2+}$, required for nuclease P1 activity, additional minor cleavage sites appeared for the wild-type topoisomerase I, but the major cleavage site (marked with an arrow in Fig. 7) was the same as the unique cleavage site seen in the absence of Mg$^{2+}$. In agreement with the results from the gel mobility shift assay, protection of the oligonucleotide from nuclease P1 digestion by the mutant topoisomerase could be observed, although several fold higher concentrations of the G194R and G194A mutant topoisomerases were required to observe comparable protection of the DNA around the major cleavage site from nuclease P1 digestion (Fig. 7). There was a difference in the nuclease P1 digestion patterns in the presence of the mutant topoisomerase I when compared with the wild-type topoisomerase. Digestion by nuclease P1 at the site marked with an asterisk in Fig. 7 was enhanced upon binding of the G194R and G194A mutant enzymes but not from the binding of the wild-type topoisomerase. Cleavage at this site remained high, whereas other nuclease P1 sites were protected by the binding of the mutant enzymes.

The ability of the G194R and G194A mutant enzymes to bind to the DNA substrate was also compared with DNase I protection using a 59-base oligonucleotide that can form a self-complementary double-stranded region. The preferred topoisomerase I cleavage site on this substrate is near the junction of the single- and double-stranded region as described previously (15). Protection from DNase I digestion by the wild-type and Gly-194 mutant enzymes was very similar indicating that the mutant enzymes could bind to the region around the preferred topoisomerase I cleavage site (Fig. 8). Again there was little or no DNA cleavage observed for the Gly-194 mutant topoisomerases showing that the step affected by the loss of Gly-194 is between the transition from the non-covalent to the covalent enzyme-DNA complexes.

Comparison of the G194R and G194A Mutants by Limited Proteolysis—Limited proteolysis with Glu-C endoproteinase was carried out with wild-type topoisomerase I and these two mutants to determine whether a drastic change in protein folding might account for the reduction in DNA cleavage activity observed. The proteolysis rate and pattern of the G194A mutant were very similar to those of the wild-type enzyme (Fig. 9) indicating that this mutation had a very limited effect on the overall folded structure of this mutant protein, despite the reduction in non-covalent interaction with the oligonucleotide substrate. The G194R mutant was digested at a faster rate by Glu-C than the wild-type topoisomerase I, and there were some differences in the pattern of proteolytic products generated.

Intrinsic Protein Fluorescence—Intrinsic fluorescence from the tryptophan residues in the topoisomerase I molecule was measured to further assess the change in enzyme folding conformation attributable to the Gly-194 mutation. The intrinsic fluorescence spectra of the wild-type topoisomerase I and the G194A and G194R mutants were found to be very similar (Fig. 10a) suggesting that these mutations did not drastically alter
the environments of the 10 tryptophan residues in the enzyme. The decrease in intrinsic fluorescence from the G194R mutation was greater than that of the G194A mutation, which is in agreement with the results of the limited proteolysis by Glu-C. The more bulky substitution with arginine probably had a greater effect on the folding of the enzyme than the alanine substitution.

When the wild-type topoisomerase I was titrated with negatively supercoiled pBR322 plasmid DNA, a significant increase in the intrinsic protein fluorescence was observed (Fig. 10b). A fluorescence emission increase of 34% was achieved with 1 nM pBR322 DNA. Higher concentrations of DNA resulted in up to 44% increase in fluorescence emission (Fig. 10f). With the G194R mutant, no fluorescence emission increase could be observed upon an addition of up to 5 nM pBR322 DNA (Fig. 10, d and f). For the G194A mutant, a small (<10%) increase of fluorescence emission upon the addition of DNA could sometimes be observed in an individual experiment (Fig. 10c), but a consistent increase in fluorescence could not be found when the data from three individual experiments were analyzed for concentrations of pBR322 DNA up to 5 nM (Fig. 10f).

To demonstrate that the change in fluorescence observed for the wild-type topoisomerase I precedes the DNA cleavage step, the topoisomerase I mutant with the active-site tyrosine mutated to phenylalanine was also analyzed. Its intrinsic fluorescence was found to be similar to that of the wild-type enzyme (Fig. 10a). Upon the addition of pBR322 DNA, there was also an increase in intrinsic fluorescence emission at about half of the magnitude observed for the wild-type enzyme (Fig. 10c and f). Therefore this active site mutant upon binding to supercoiled DNA could also undergo a conformational change leading to the increase in fluorescence even though it cannot form the covalent complex with DNA. In contrast, the G194R and G194A mutants were found to be defective for this conformational change.

**DISCUSSION**

A number of conserved amino acids around the active site of *E. coli* DNA topoisomerase I have been shown by site-directed mutagenesis studies (18–20) to be involved in the DNA cleavage step of catalysis by the enzyme. The proposed roles for these conserved amino acids during DNA cleavage include binding of the scissile phosphate, stabilization of the transition state, and leaving group during nucleophilic attack by the active-site tyrosine. These are directly related to the chemical functions present on the side chains of these residues. How-
ever, other amino acids in the enzyme at a greater distance from the active-site tyrosine are likely to be required for the conformational changes involved in catalysis instead of direct chemical involvement in the DNA cleavage and religation. These residues that are important for enzyme conformational changes instead of the chemical steps in the enzyme mechanism remain to be identified.

Based on the crystal structure of *E. coli* topoisomerase III complexed to single-stranded oligonucleotide, the movement of an α helix was proposed to be important for the positioning of the scissile phosphate at the active site of type IA DNA topoisomerases (10). It is hypothesized here that the flexibility of the conserved Gly-194 residue at the N terminus of this α helix in *E. coli* topoisomerase I is important for its function as a hinge

![Intrinsic fluorescence of wild-type and G194R and G194A mutant topoisomerase](image-url)

**Fig. 10.** Intrinsic fluorescence of wild-type and G194R and G194A mutant topoisomerase. *a,* comparison of intrinsic fluorescence of wild-type and G194R, G194A, and Y319F mutant topoisomerases. *b–e,* effect of the addition of negatively supercoiled plasmid pBR322 DNA on the intrinsic fluorescence of wild-type and G194R, G194A, and Y319F mutant topoisomerases. *f,* plot of the change in fluorescence emission at 340 nm with the amount of DNA added.
for this movement. To assess the importance of the hypothesized hinge function, the Gly-194 of *E. coli* topoisomerase I was mutated to six other amino acids. All six substitutions were found to result in a significant loss of enzyme relaxation activity. Detailed analysis of the purified G194A and G194R mutants showed that the reduction of relaxation activity was ~40-fold for the alanine-substituted mutant, whereas no relaxation activity was retained for the more bulky arginine substitution. The loss of relaxation activity resulted primarily from the ~100-fold reduction in DNA cleavage activity. The decrease in overall non-covalent complex formation due to the mutations was relatively minor as the mutant enzymes could still form a gel mobility shift complex with the oligonucleotide substrate and protect the bound DNA from P1 nuclease and DNase I digestion. There was some difference in protein folding between the wild-type enzyme and the G194R mutant with the glycine substituted by the more bulky arginine, but the more conservative G194A mutation appeared to have little effect on the enzyme structure in the absence of DNA, as assessed by limited proteolysis and intrinsic fluorescence measurement. A comparison of the response of the intrinsic fluorescence upon the addition of negatively supercoiled DNA showed that the wild-type topoisomerase underwent a conformational change affecting the environment of the tryptophan residues. A similar increase in intrinsic protein fluorescence was also observed for the active-site Y319F mutant topoisomerase I demonstrating that the conformational change leading to the fluorescence increase preceded the formation of the cleaved covalent complex. This increase in intrinsic fluorescence was not detected with the Gly-194 mutant topoisomerases, in agreement with the Gly-194 residue being responsible for a conformational change that takes place after an initial non-covalent binding to DNA and before DNA cleavage takes place. Therefore, in the non-covalent complex formed by the G194R and G194A mutants, the DNA substrate was likely not positioned correctly for nucleophilic attack to take place on the scissile phosphate by the active-site tyrosine.

The sensitivity of enzyme cleavage and relaxation activity to substitution at Gly-194 supports the importance of this residue in the overall topoisomerase I mechanism. There are many known examples of critical glycine residues acting as hinges in the enzyme conformational change required for catalysis (21–24). For example, a glycine to arginine mutation in the hinge region of *E. coli* tryptophan synthase also blocked the conformational switching (21). A glycine to tryptophan mutation was used to demonstrate the role of the glycine in the conformational change of human O-6-alkylguanine DNA-alkyltransferase upon DNA binding (22). Even though the Gly-194 of *E. coli* DNA topoisomerase I is located at a considerable distance from the active-site tyrosine, its substitution by another amino acid could still affect the DNA cleavage step drastically. It is likely to be required for shifting the α helix containing Arg-195 and Gin-197 in place to interact with the DNA substrate specifically so that the scissile phosphate could be properly aligned for nucleophilic attack by Tyr-319. The Gly-132 of vaccinia DNA topoisomerase I located at an interdomain hinge is invariant among type IB DNA topoisomerases (25). Mutagenesis results obtained previously (25) suggested that this glycine residue in vaccinia DNA topoisomerase I is required for a precleavage activation step. This is similar to the role proposed for the Gly-194 of *E. coli* DNA topoisomerase I in this study.

This conformational change involving Gly-194 as the hinge is only one of the conformational changes likely to be required for a complete cycle of linking number change catalyzed by *E. coli* DNA topoisomerase I. After DNA cleavage takes place, the spacing between the 5'-phosphate and 3'-hydroxyl groups of the cleaved DNA must be increased to allow DNA strand passage, and then the spacing had to be decreased again to allow DNA religation to take place. The mechanisms of these conformational changes remain to be elucidated.

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