Site-directed Mutagenesis of Residues Involved in G Strand DNA Binding by Escherichia coli DNA Topoisomerase I*

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Crystal structures of complexes between type IA DNA topoisomerases and single-stranded DNA suggest that the residues Ser-192, Arg-195, and Gln-197 in a conserved region of Escherichia coli topoisomerase I may be important for direct interactions with phosphates on the G strand of DNA, which is the substrate for DNA cleavage and religation (Changela A., DiGate, R. J., and Mondragón, A. (2001) Nature 411, 1077–1081; Perry, K., and Mondragón, A. (2002) Structure 11, 1349–1358). Site-directed mutagenesis experiments altering these residues to alanines and other amino acids were carried out to probe the relevance of these interactions in the catalytic activities of the enzyme. The results show that the side chains of Arg-195 and Gln-197 are required for DNA cleavage by the enzyme and are likely to be important for positioning of the G strand of DNA at the active site prior to DNA cleavage. Mutation of Ser-192 did not affect DNA binding and cleavage but nevertheless decreased the overall rate of relaxation of supercoiled DNA probably because of its participation in a later step of the reaction pathway.

DNA topoisomerases are ubiquitous enzymes that catalyze the interconversion of different DNA topological forms by the concerted breaking and rejoining of one or two DNA strands; these are coupled to the DNA strand passage through the break created. The various families of type I and II DNA topoisomerases play important roles in vital cellular processes including replication, transcription, and recombination (1–3). Escherichia coli DNA topoisomerase I is a type IA DNA topoisomerase responsible for removal of excess negative supercoils from the bacterial DNA (4–6). In each catalytic cycle of the proposed enzyme-bridging mechanism, the enzyme binds and cleaves a single strand of DNA (denoted as G strand here), forming a covalent 5'-phosphotyrosine linkage to DNA, to create a “gate” for subsequent strand passage (7, 8). A number of residues proximal to the active site tyrosine have been implicated by previous biochemical and genetic experiments as important for interacting with the scissile phosphate in the DNA cleavage step. However, contacts with other phosphates on the DNA are also likely to be important in the mechanism of the enzyme because sequence-specific recognition of DNA bases does not appear to be an overriding factor in interaction of the enzyme with the DNA substrate. These interactions may be important for catalytic stages of the enzyme either prior to or after the nucleophilic attack on the scissile phosphate.

Important insights into the potential enzyme-phosphate contacts have come from several crystal structures of E. coli topoisomerase I and III. In the crystal structure of the 67-kDa N-terminal fragment of the enzyme, the tyrosine responsible for DNA cleavage (Tyr-319) is not accessible for interaction with the DNA substrate (9). This structure probably represents a closed conformation of the enzyme. From this closed conformation, the enzyme is expected to undergo conformational changes, first to allow the G strand DNA to bind to the enzyme, and second to position the hydroxyl group of Tyr-319 for nucleophilic attack on a scissile phosphate on the bound G strand DNA. The enzyme-DNA complex poised for DNA cleavage should resemble the complex formed between single-stranded DNA and E. coli topoisomerase III with the active site tyrosine mutated to phenylalanine (10). In this topoisomerase III-ssDNA complex (Fig. 1a), the side chains of residues corresponding to Arg-195 and Gln-197 of E. coli topoisomerase I make contacts with the DNA phosphodiester backbone at two of the phosphates 5' but not directly adjacent to the scissile phosphate (10). In a structure of the N-terminal domain of E. coli topoisomerase I (with H365R substitution) complexed to single-stranded DNA, the active site is not formed (11). Nevertheless the ssDNA occupies the groove leading to the active site (Fig. 1b), and it was postulated that this structure represents an intermediate conformational state in the catalytic cycle (11). In this structure, Gln-197 and Ser-192 contact the phosphodiester backbone, but Arg-195 does not make any DNA contacts (Fig. 1). Arg-195 and Gln-197 are located at the N-terminal region of an α helix that has shifted significantly in the topoisomerase III-ssDNA structure (10), and Ser-192 is found in the loop leading to this shifted α helix. These three residues are part of a highly conserved region among type IA DNA topoisomerase sequences that extends from Ser-192 to Ser-198 (SARGVQS), with Ser-192 and Gln-197 both being invariant (11, 12). In contrast, many other residues observed to make contacts with the bound DNA are not highly conserved (10). However, there is a lack of direct biochemical or genetic evidence supporting the roles of these conserved residues in the relaxation of DNA by the enzyme. The contacts between Ser-192, Arg-195, Gln-197, and the DNA phosphodiester backbone away from the active site tyrosine may be important for the initial binding and/or positioning of the G strand of DNA at the active site for DNA cleavage during the catalytic cycle. To test this hypothesis, site-directed mutagenesis was carried out to investigate the effects of the single substitutions on enzyme activity.

EXPERIMENTAL PROCEDURES

Enzymes—Wild-type and mutant E. coli DNA topoisomerase I proteins were expressed and purified as described previously (13). Plasmid-expressing mutant topoisomerase I was generated from

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The abbreviation used is: ssDNA, single-stranded DNA.
plasmid pRV10 (14) as a template using Pfu ultra DNA polymerase I (from Stratagene). The topoisomerase I gene in this plasmid is under the control of the lacUV5 promoter, and the lacI gene is also present on the plasmid to reduce the basal level of expression. The primers used in the mutagenesis were synthesized by Sigma-Genosys, and the sequences of the top strand oligonucleotides with the mutated bases underlined were 5′-GCTGTCGCGCGTGTGACCCGGTGGTGG-3′ (S192A), 5′-CGTCTGTCGCGCGTGTGACCCGGTGGTGG-3′ (S192T), 5′-CTGTCTGCGCGCGTGTGACCCGGTGGTGG-3′ (R195A), 5′-CTGTCTGCGCGTGTGACCCGGTGGTGG-3′ (R195K), 5′-GCGGCGGCGGTGTGACCCGGTGGTGG-3′ (Q197A).

**Non-covalent Interaction between the enzyme and the oligonucleotide**

**Non-covalent Binding Assay**—Non-covalent interaction between the enzyme and the oligonucleotide used in the cleavage reaction was determined by the gel electrophoretic mobility shift assay. The wild-type and mutant topoisomerase I was incubated with 1 pmol of the oligonucleotide in a 10-μl reaction mixture of 20 mM Tris-HCl, pH 7.5, 0.1 mM dithiothreitol, and 50% (v/v) glycerol, and 0.5% (v/v) bromphenol blue. The reaction products were analyzed by electrophoresis in a 0.8% (w/v) agarose gel with TAE buffer (40 mM Tris acetate, pH 8.1, 2 mM EDTA). The Alpha-Imager was used to record the image of the gel stained with ethidium bromide.

**Limited Proteolytic Digestion**—The wild-type and mutant topoisomerase I proteins were subject to limited digestion by the Glu-C endoproteinase (sequencing grade from Roche Applied Sciences) with 5 μg of the protein digested at a ratio of 1:100 in 20 mM potassium phosphate, pH 7.5, 20 mM KCl, 0.2 mM dithiothreitol for the indicated lengths of time. After mixing with equal volume of 2× SDS gel loading buffer and boiled for 5 min, the digestion mixtures were analyzed by SDS-PAGE in a 15% gel. The digestion products were visualized by Coomassie Blue staining.

**In Vivo Complementation by the Topoisomerase I Mutants**—The ability of these mutants to complement topA function in E. coli was tested after transformation of the expression plasmid derived from pRV10 into strain AS17 (F′ topA(am) pLL1[Tet(supD43,74)]) with a temperature-sensitive topoisomerase I protein encoded on the chromosome (18, 19). After overnight growth at 30 °C, dilutions of the transformants were plated on LB agar plates containing ampicillin and 2% glucose so that only a low level of recombinant topoisomerase I was expressed from the plasmid. After incubation at 30 and 42 °C, the ratios of colonies at the
two temperatures were determined and compared with the plasmid expressing the wild-type topoisomerase I.

RESULTS

Substitutions of Ser-192, Arg-195, and Gln-197 Resulted in Loss of Relaxation Activity—To test the requirement of the side chains of these residues for the relaxation activity of E. coli DNA topoisomerase I, the residues were mutated to alanines. The resulting mutant enzymes were purified and analyzed for relaxation activity for 30 min in a standard reaction buffer with 50 mM NaCl. The results (Fig. 2) showed that the alanine substitutions resulted in the most significant loss of activity at the Arg-195 position with relaxation activity reduced by 500-fold, followed by the Gln-197 substitution (100-fold). The alanine substitutions at Ser-192 resulted in 10–20-fold lower activity. Substituting Arg-195 with the similarly charged lysine also had a significant effect on the relaxation activity (50–100-fold loss). The presence of a threonine with a hydroxyl side chain at position 192 improved the activity over the alanine substitution mutant to about one-tenth of the wild-type activity.

DNA Cleavage Is Affected by Substitutions at Arg-195 and Gln-197—We next determined the effect of the substitutions on cleavage of a 203-base-long single-stranded DNA (Fig. 3). Whereas the cleavage products for the R195A,
R195K, and Q197A mutants were severely reduced, there was little change in the amount of cleavage products formed by the S192A and S192T mutant enzymes. The effect of the mutations on DNA cleavage by topoisomerase I was also measured using a shorter oligonucleotide substrate (Fig. 4). Again the cleavage rates of the S192A and S192T mutant enzymes were about the same as the wild-type topoisomerase. No cleavage could be detected for the R195A mutant enzyme (data not shown), and the cleavage rates for the R195K and Q197A mutants were about 20–40-fold lower than the wild-type topoisomerase.

The R195A and Q197A Mutant Enzymes Had Reduced Non-covalent Binding to DNA—The non-covalent binding of the mutant topoisomerase to the oligonucleotide substrate used in the cleavage assay was assessed using the gel mobility shift assay. The results (Fig. 5) showed that the S192A, S192T, and R195K mutant enzymes had a ~2-fold change in non-covalent binding to the DNA substrate. The level of gel shift complex formed by the R195A and Q197A mutant enzymes was about 3–4-fold lower than that of the wild-type topoisomerase.

Substitutions at Ser-192 Have No Effect on DNA Rejoining—There was no apparent effect from the substitutions at Ser-192 on the DNA cleavage activity of topoisomerase I. To determine whether deficiency in DNA rejoining affected the relaxation activity of the S192A and S192T mutants, DNA rejoining was assayed by first forming the cleaved complex with the 203-base-long single-stranded DNA labeled at the 5’ end (lane 1, Fig. 6). The cleaved complex is salt-stable (20), so addition of 1 M NaCl alone to the reaction mixture had no effect on the amount of DNA cleavage product (lane 2, Fig. 6). Addition of 4 mM MgCl₂, however, resulted in the rejoining of the cleaved DNA and dissociation of enzyme in the presence of the high salt (lane 3, Fig. 6). The rejoining of the cleaved DNA by the S192A and S192T mutant enzymes was just as efficient as the wild-type topoisomerase I (Fig. 6), which indicated that the substitutions had no effect on this step of enzyme activity in the relaxation mechanism. The rejoining kinetics was very rapid and completed within 30 s of the addition of salt and Mg(II) for both the wild-type and the Ser-192 mutant enzymes (data not shown).

The R195K Had a More Significant Change in Structure as Determined by Limited Proteolysis—Limited proteolysis with the Glu-C endoproteinase was used to compare the extent to which the enzyme structure might have been perturbed by mutations. The results (Fig. 7) showed that the digestion pattern of the R195K mutant had the greatest change when compared with the digestion pattern of the wild-type enzyme. The digestion patterns of the other mutant topoisomerases were quite similar to those of the wild-type enzyme.

In Vivo Activity of the Mutant Topoisomerases—The in vivo...
activity of these mutant topoisomerases was measured by their ability to complement the temperature-sensitive topoisomerase I activity encoded by the chromosomal topA of strain AS17 (18, 19). The number of colonies formed in the presence of the recombinant wild-type or mutant topoisomerase I was compared at 30 and 42 °C. Whereas the wild-type recombinant topoisomerase I could fully support growth at 42 °C, the number of viable colonies observed for the mutant topoisomerases at 42 °C was greater than one thousand fold less than that observed at 30 °C (Table I). The effects of the mutations on the in vivo topoisomerase activity required for viability account for the high degree of conservation of these amino acids among type IA topoisomerase sequences (11, 12).

The Activity of the Mutant Enzymes in the Presence of 0.2 M Potassium Glutamate—The relaxation activities of the wild-type and mutant topoisomerases were also assayed in a buffer containing glutamate instead of chloride as the counterion to better imitate the intracellular ionic conditions in E. coli (21, 22). The results (Fig. 8a) showed that the wild-type topoisomerase I relaxation activity did not decrease significantly when the 50 mM NaCl in the in vitro assay buffer was replaced with 0.2 M potassium glutamate. As intracellular glutamate concentration can be greater than 0.25 M depending on the external osmolarity (21, 22), we compared the time course of relaxation by the mutant and wild-type topoisomerase I in the presence of 0.2 M potassium glutamate (Fig. 8b). All the mutant enzymes examined here could not complete the relaxation of the negatively supercoiled DNA even when present at a relatively high concentration (200 nM).

### Table I

| Recombinant topoisomerase I | Ratio of colonies at 42 °C versus 30 °C |
|-----------------------------|----------------------------------------|
| Wild-type                   | 1.0                                    |
| S192A                       | 2.4 × 10⁻⁵                             |
| S192T                       | 1.4 × 10⁻⁴                             |
| R195A                       | 1.3 × 10⁻⁵                             |
| R195K                       | 5.5 × 10⁻⁴                             |
| Q197A                       | 3.7 × 10⁻⁴                             |
| None                        | <10⁻⁶                                  |

![Fig. 8](http://www.jbc.org/)

Fig. 8. a, relaxation activity of wild-type topoisomerase I in 200 mM potassium glutamate (replacing the 50 mM NaCl) in the relaxation buffer after 30 min of incubation. b, time course of relaxation with 200 mM potassium glutamate in the relaxation buffer.
The DNA binding and cleavage activities of the Ser-192 mutants were also examined in the presence of 0.2 mM glutamate (Fig. 9). At this higher ionic strength, a 2-fold reduction in DNA binding was observed for the S192A mutant with the gel electrophoretic mobility shift assay. Nevertheless, even at this higher ionic strength that may more accurately resemble the in vivo conditions, there did not appear to be a significant effect on the DNA cleavage activity from the mutation at this position.

DISCUSSION

Previous site-directed mutagenesis experiments from different laboratories have characterized a number of conserved residues in E. coli DNA topoisomerase I that were proposed to be interacting with the scissile phosphate in the DNA cleavage mechanism (23–25). These residues include Tyr-319, Arg-321, and Glu-9. In addition, it was proposed that His-365 participates in a proton relay mechanism so that Glu-9 can donate a proton to the leaving 3'-oxygen of the scissile phosphate (25).

The acidic triads of Asp-111, Asp-113, and Glu-115 have been implicated by crystallographic studies in the relaxation activity (24, 26). Residues that interact with other phosphates on the DNA cleavage substrate have been postulated to correspond to an intermediate conformation between the closed state (9) and the catalytically competent state (11). Ser-192 was observed to contact a phosphate. Results from this study showed that although this phosphate interaction did not appear to be required for DNA cleavage to take place, mutation of Ser-192 could still have a significant effect on the overall relaxation reaction rate, both in vitro and at higher ionic strength conditions in vivo. It was unclear from the crystal structure whether the complex observed corresponds to a precleavage or to a postligation structure of the enzyme although it was postulated that the complex is representative of both stages (11). The results obtained here suggested that the direct interaction observed for Ser-192 in this crystal structure was not required for events leading to DNA cleavage. Nevertheless, this residue is highly conserved, and its mutation did have a significant effect on the relaxation activity. This is unlikely to be due to the effect of the mutations on protein folding, as the mutant enzymes retained the wild-type level of DNA cleavage activity. The alanine substitution at position 195 might be able to participate in an electrostatic interaction with DNA, but it could not position the G strand DNA correctly for DNA cleavage to take place, perhaps because of the change in enzyme folding caused by the arginine to lysine substitution, as indicated by the proteolytic digestion data. The side chains of residues corresponding to Arg-195 and Gln-197 in E. coli DNA topoisomerase III were observed in the complex with single-stranded DNA to be in contact with the phosphodiester backbone (10). The side chain of the highly conserved arginine interacts with the phosphate two nucleotides upstream of the scissile phosphate whereas the side chain of the invariant glutamine interacts with two phosphates three and four nucleotides upstream of the scissile phosphate simultaneously (10). These interactions are likely to assist the positioning of the scissile phosphate for nuclease attack by the tyrosine nucleophile. The arginine and the glutamine are at the base of an α helix that had shifted its position when the crystal structures of the closed topoisomerase alone (27) and the catalytically competent topoisomerase-asDNA complex of E. coli DNA topoisomerase I (16). The results here provided biochemical evidence that the protein-DNA interactions involving Arg-197 and Gln-199 observed in the crystal structure of the catalytically competent topoisomerase III-asDNA complex are important for DNA cleavage to take place. In addition, the phosphate interactions by Arg-197 and Gln-199 may also be important in the movement of the DNA binding domains after DNA cleavage, so that the cleaved DNA ends could be physically separated to create space for strand passage, as discussed recently (29).

The serine at position 192 of E. coli DNA topoisomerase I is even more highly conserved among type IA DNA topoisomerases than the arginine at position 195 (11, 12). In the structure of the complex between E. coli DNA topoisomerase I and the single-stranded DNA postulated to correspond to an intermediate conformation between the closed state (9) and the catalytically competent state (11), Ser-192 was observed to contact a phosphate. Results from this study showed that although this phosphate interaction did not appear to be required for DNA cleavage to take place, mutation of Ser-192 could still have a significant effect on the overall relaxation reaction rate, both in vitro and at higher ionic strength conditions in vivo. It was unclear from the crystal structure whether the complex observed corresponds to a precleavage or to a postligation structure of the enzyme although it was postulated that the complex is representative of both stages (11). The results obtained here suggested that the direct interaction observed for Ser-192 in this crystal structure was not required for events leading to DNA cleavage. Nevertheless, this residue is highly conserved, and its mutation did have a significant effect on the relaxation activity. This is unlikely to be due to the effect of the mutations on protein folding, as the mutant enzymes retained the wild-type level of DNA cleavage activity. The alanine substitution at Ser-192, and to a lesser extent, the threonine substitution, could have a cumulative effect on the ability of the enzyme to go through the transitions from the ligated complex after each DNA strand passage event to a conformation required for the cycle of catalysis to repeat again. That might account for the overall effect of the mutation on the DNA relaxation activity. Alternatively, this interaction may be involved in the movement of the DNA binding domains needed for strand passage to take place.

In summary, the results here showed that these three con-
served amino acids in *E. coli* DNA topoisomerase I, although likely not involved directly in the chemistry of DNA cleavage and religation at the active site, could still be playing important roles in the different stages of the catalytic mechanism of the enzyme as they guide the protein-DNA interactions through the transitions of the different complexes required for change in DNA topology.

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