Site-directed Mutagenesis of Conserved Aspartates, Glutamates and Arginines in the Active Site Region of Escherichia coli DNA Topoisomerase I

(Received for publication, September 15, 1997, and in revised form, December 21, 1997)

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DNA topoisomerases (for review, see Refs. 1–7) catalyze the interconversion of different DNA topological isomers by first forming a covalent enzyme-DNA intermediate via nucleophilic attack of a tyrosine hydroxyl group on the DNA phosphodiester backbone bond during the step of DNA cleavage. Strand passage then takes place to change the linking number. This is followed by DNA religation during which the displaced DNA hydroxyl group attacks the phosphotyrosine linkage to reform the DNA phosphodiester bond. Mg(II) is required for the relaxation activity of type IA and type II DNA topoisomerases. A number of conserved amino acids with acidic and basic side chains are present near Tyr-319 in the active site of the crystal structure of the 67-kDa N-terminal fragment of Escherichia coli DNA topoisomerase I. Their roles in enzyme catalysis were investigated by site-directed mutation to alanine. Mutation of Arg-136 abolished all the enzyme relaxation activity even though DNA cleavage activity was retained. The Glu-9, Asp-111, Asp-113, Glu-115, and Arg-321 mutants had partial loss of relaxation activity in vitro. All the mutants failed to complement chromosomal topA mutation in E. coli AS17 at 42 °C, possibly accounting for the conservation of these residues in evolution.

To investigate the roles of the carboxylates and arginine residues in the active site region of E. coli topoisomerase I, they were altered by site-directed mutagenesis to alanines, abolishing the acidic or basic functional groups. The mutant enzymes were expressed and purified. Different enzymatic assays were carried out to determine how the mutation affected the interaction of the topoisomerase enzyme with DNA and/or Mg(II).

EXPERIMENTAL PROCEDURES

Materials—All chemical reagents used were ultrapure or Baker analyzed reagent grade. Solutions were prepared with water first deionized with the Barnstead Nanopure system and then passed over a Bio-Rad Chelex 100 resin (100–200 mesh sodium form) to remove any remaining contaminating metal ions. Tubes, spectrophotometric cells, and glassware for metal ion-sensitive experiments were first washed with 10 mM EDTA and then rinsed extensively with metal-free water.

* This work was supported by Grant GM54226 from NIGMS, National Institutes of Health, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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FIG. 1. Alignment of type IA topoisomerase sequences to demonstrate the conservation of amino acid residues (highlighted) mutated in this study. ecl, *Escherichia coli* topoisomerase I (33); hii, *Hemophilus influenzae* topoisomerase I (34); bsi, *Bacillus subtilis* topoisomerase I (Swiss Protein accession number P38984); mtI, *Mycobacterium tuberculosis* topoisomerase I (35); tml, *Thermotoga maritima* topoisomerase I (36); mtI, *Mycoplasma genitalium* topoisomerase I (37); mpI, *Mycoplasma pneumoniae* topoisomerase I (38); mjI, *Methanococcus jannaschii* topoisomerase I (39); ecIII, *Escherichia coli* topoisomerase III (40); hiIII, *Hemophilus influenzae* topoisomerase III (41); ScIII, *Saccharomyces cerevisiae* topoisomerase III (42); HII, *H. influenzae* topoisomerase III (43); saR, *Sulfobolus acidocaldarius* reverse gyrase (44); mkr, *Methanopyrus kandleri* reverse gyrase (45); mjR, *Methanococcus jannaschii* reverse gyrase (39).

Mutagenesis—Plasmid pJW312, with the *topA* coding region under the control of the lac promoter (21), was used as the template for mutagenesis. The D111A, R136A, and R321A mutants were constructed with the Chameleon site-directed mutagenesis kit, also from Stratagene while the E9A, D113A, and E115A mutants were constructed with the QuikChange site-directed mutagenesis kit, also from Stratagene. The D111A, R136A, and R321A mutants were constructed with the Chameleon site-directed mutagenesis kit from Stratagene while the E9A, D113A, and E115A mutants were constructed with the QuikChange site-directed mutagenesis kit, also from Stratagene.

Enzyme Expression and Purification—Wild-type *E. coli* DNA topoisomerase I enzyme was expressed from *E. coli* MV1190 cells transformed with plasmid pJW312. The R136A and R321A mutants were expressed in *E. coli* AS17 (topAcysB)204, from R. E. Depew, Northeastern Ohio University). The E9A, D111A, D113A, and E115A mutants were constructed with the QuikChange site-directed mutagenesis kit, also from Stratagene while the E9A, D113A, and E115A mutants were constructed with the Chameleon site-directed mutagenesis kit from Stratagene.

Relaxation Activity Assay—Wild-type and mutant enzymes were serially diluted and assayed for relaxation activity in 20 μl with 0.5 μg of negatively supercoiled plasmid DNA, 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, and 6 mM MgCl2 unless indicated otherwise. Incubation was at 37 °C for 30 min. The reactions were stopped by the addition of 5 μl of 50% glycerol, 50 mM EDTA, and 0.5% (v/v) bromphenol blue. After electrophoresis in a 6% DNA sequencing gel, the 5'-end-labeled DNA was visualized by autoradiography of the dried gel.

Cleavage of 5'-end-labeled Single-stranded DNA—Plasmid p7T−1 DNA (from U. S. Biochemical Corp.) was cleaved with EcoRI and then labeled at the 5'-end with [γ-32P]dATP and T4 polynucleotide kinase. The labeled DNA was denatured to single strand and incubated with the wild-type and mutant enzymes, and then sodium hydroxide was added to trap the covalent complex and cleavage of the DNA (24). After electrophoresis in a 6% DNA sequencing gel, the 5'-end-labeled DNA cleavage products were visualized by autoradiography.

Covalent Complex with Oligonucleotide Substrate—The single-stranded oligonucleotide 5'-CAATGCGCTA-CATCGGTGGTTTAAAC-3' (R316A) and 5'-TATACCTAATCGCTACCCATCCAC-3' (R321A). The mutants were identified by DNA sequencing of the plasmid DNA.

FIG. 2. SDS gel of purified *E. coli* DNA topoisomerase I wild-type (WT) enzyme and mutants with the indicated residues changed to alanine.

**MW (kD)**

| WT | D111E | D113A | E115A |
|----|-------|-------|-------|
| 205 | 116   | 97    | 66    |
| 55  |       |       |       |
| 45  |       |       |       |
| 36  |       |       |       |

**SDS**

| E9A | D111A | D113A | E115A |
|-----|-------|-------|-------|
| 111  | 97    | 66    |       |
| 55  |       |       |       |
| 45  |       |       |       |
| 36  |       |       |       |

**Tyr319**

| Arg316 | Arg321 |
|--------|--------|
| 319    | 321    |

**Glu9**

| Asp111 | Asp113 | Glu115 |
|--------|--------|--------|
| 117    | 123    | 88     |
| 101    | 109    | 90     |
| 108    | 109    | 90     |
| 107    | 109    | 90     |

**Tables:**

| Arg136 | Tyr319 | Arg321 |
|--------|--------|--------|
| 324    | 330    | 303    |
| 352    | 293    | 339    |
| 345    | 320    | 332    |
| 342    | 342    | 342    |

**Figure:**

A gel mobility shift assay was carried out using the previously described procedures (23) with an additional hydroxyapatite chromatography step between the phosphocellulose and single-stranded DNA-agarose columns.

Before use. Plasmid DNA was purified by cesium chloride centrifugation.
tion was carried out using the Molecular Dynamics PhosphorImager.

Intrinsic Tryptophan Fluorescence Measurements—Fluorescence measurements were performed with the Perkin-Elmer LS-5B spectrometer with excitation at either 280 or 295 nm at either 42 °C or room temperature (25 °C). The spectral bandwidths were 3 and 10 nm, respectively, for excitation and emission. The wild-type or mutant topoisomerase I was present at 0.1 mg/ml in 20 mM potassium phosphate, pH 7.5, 0.1 M KCl, 0.2 mM dithiothreitol. All the measurements were corrected for the spectrum of the buffer used.

Equilibrium Dialysis to Determine Mg(II) Binding Stoichiometry—1 ml of topoisomerase I (0.4 mg/ml) was dialyzed against 400 ml of buffer (20 mM potassium phosphate, pH 7.5, 0.1 M KCl, 0.2 mM dithiothreitol, and 400 μM MgCl2) at room temperature for 7 h. The enzyme and dialysis buffer samples were submitted to Quantitative Technologies, Inc., NJ, for Mg(II) content analysis using the inductively coupled plasma method.

RESULTS

Expression and Purification of the Topoisomerase I Mutants—After site-directed mutagenesis of the plasmid pJW312 to produce the desired alanine substitutions, expression of the mutant proteins in E. coli strain AS17 was examined by SDS-gel electrophoresis of the total soluble extract followed by Coomassie Blue staining of the gel. The expression level of the Arg-136 mutant was comparable with that of the wild-type topoisomerase I while expression of the Arg-321 mutant was detectable but lower than the wild type. Bands corresponding to the Glu-9, Asp-111, Asp-113, and Glu-115 mutants could not be identified in AS17 extracts (data not shown). Expression of these three mutant enzymes were then carried out in E. coli strain GP200. Detectable levels of expression of these mutant enzymes allowed the purification of the mutant enzymes to homogeneity (Fig. 2).

Effect of the Mutations on Enzyme Activities—Purified mutant enzymes were diluted serially and compared with the wild-type topoisomerase I for relaxation of negatively supercoiled plasmid DNA in relaxation buffer containing 6 mM MgCl2 (Fig. 3A). The results showed that the Arg-136 mutant was totally inactive in the relaxation assay. No relaxation activity was observable even with 400 ng of the enzyme, a 50-fold excess over the amount needed to observe relaxation by the wild-type enzyme. The other mutants had varying degrees of loss of relaxation activity. Examination of the effect of dilutions and time course of relaxation (Fig. 3B) showed that the Glu-9 mutant had the greatest reduction in catalytic activity (<90% reduction). The Glu-115, Asp-113, and Arg-321 mutants had about 80–90% reduction in activity. The Asp-111 mutant was closest to the wild-type enzyme in activity (<50% reduction).

The E. coli strain AS17 does not grow at 42 °C because of the
temperature sensitivity of the suppressor for the chromosomal topA
mut mutation. The pJW312 plasmid carrying the wild-type
topoisomerase I gene can complement this chromosomal muta-
tion (21). Each one of the active site mutations tested here was
found to abolish this in vivo complementation even though they
showed varying degrees of loss of in vitro relaxation (Table I).

The inability of the Glu-9, Glu-111, Asp-113, and Glu-115
mutants to complement efficiently in E. coli AS17 may be due
to their low level of expression in this E. coli strain. To evaluate
the in vivo activities of these mutants, the thermosensitivities
of GP200 expressing these mutants were examined (Table II).
Loss of topA activity in E. coli can lead to a lower rate of
survival when the temperature was raised to 52 °C (reviewed
in Ref. 25). The rate of survival of E. coli GP200 was increased
50-fold when plasmid encoded wild-type topA activity was
present (Table II). The survival rates of GP200 transformed
with plasmid encoding the Glu-9, Glu-111, Asp-113, and Glu-
115 mutants correlated with their in vitro activity, with the
Glu-111 mutant conferring close to wild-type thermoresistance
and the Glu-9 mutant conferring the least amount of
thermoresistance.

The Arg-136 Mutant Could Cleave DNA and Form the Covalent
Complex—The cleavage activities of the mutant enzymes
were examined using 5'-end-labeled single-stranded DNA. Even
though the Arg-136 mutant was totally inactive in the
relaxation assay, the amount of cleaved DNA formed was compar-
able with that from the wild-type enzyme (Fig. 4). For the
other mutants, the amounts of cleavage products observed
were decreased, with the Glu-9 mutant having the lowest
cleavage activity, so that the reduction of relaxation activity
seen with these other mutants might be due to the decrease in
the amount of cleaved complex formed by these mutants. The
Asp-111 mutant had the same DNA cleavage efficiency as the
wild-type enzyme.

A 3'-end-labeled oligonucleotide substrate was used to form
a covalent complex with the enzyme. At 5 min after the addi-
tion of the enzyme to the oligonucleotides, the amounts of the
covalent complex observed for the wild-type and the Arg-136
mutant were identical (Fig. 5). The amount of covalent complex
formed by the Asp-111 mutant was also close to that of the wild
type while the other mutant enzymes gave lower levels of

| Plasmid-encoded topoisomerase I | Survival rate |
|---------------------------------|--------------|
| Wild-type                       | 0.35         |
| E9                              | 0.040        |
| D111A                           | 0.17         |
| D113A                           | 0.077        |
| E115A                           | 0.050        |
| None                            | 0.006        |

FIG. 4. Cleavage of 5'-end-labeled single-stranded DNA by
wild-type and mutant E. coli topoisomerase I enzymes. The cleav-
age reactions were analyzed by electrophoresis in a 6% DNA sequencing
gel. C, no enzyme added.

FIG. 6. Inter-molecular strand transfer by the wild-type topoi-
somerase I and the R136A mutant enzyme. The reaction conditions
were as described in Ref. 27, with the enzymes first incubated with the
3'-end-labeled oligonucleotide used in the covalent complex formation
assay. HindIII-digested λ DNA (0.5 μg) was then added as the acceptor
molecule. C, no enzyme added.
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labeled covalent complexes under the experimental conditions employed. This experiment also demonstrated that the Arg-136 mutation affected a step in the enzyme relaxation mechanism that took place after DNA cleavage. This could be the strand passage or the DNA religation step. The inter-molecular strand transfer activity of the Arg-136 mutant was compared with the wild type enzyme using the covalent complex formed with this 3’-end-labeled oligonucleotide substrate and HindIII-digested λ DNA as acceptor molecules (26). The result from one experiment is shown in Fig. 6. Data from several experiments indicated that the inter-molecular religation activity is about the same for the wild-type and the Arg-136 mutants. The efficiency of this reaction is low for both the wild-type and Arg-136 mutant enzymes. It may or may not reflect the relative activity of the intra-molecular religation that takes place during relaxation of supercoiled DNA.

The Glu-9, Asp-113, Glu-115, and R321 Mutations on Non-covalent DNA Binding—The decreased amount of cleaved complex formed by some of these mutants could be due to the effect of the mutation on non-covalent binding to DNA. The gel mobility shift assay was used to evaluate the non-covalent topoisomerase-DNA complex formation. The results (Fig. 7) showed that the Arg-321 and Glu-9 mutants had about 50% of the DNA binding activity as that of the wild-type enzyme. The Glu-115 mutant had slightly less binding activity (40%) while the Asp-113 mutant had the lowest DNA binding activity (25%).

The Glu-9 and Glu-115 Mutations Altered the Mg(II) Binding—The mutant enzymes were tested for Mg(II) binding by equilibrium dialysis against buffer containing 0.4 mM MgCl₂. The Mg(II) binding stoichiometry was determined by inductively coupled plasma analysis (Table III). At this Mg(II) concentration, each molecule of the wild-type enzyme has been found to bind around 2 Mg(II) (27). A higher Mg(II) binding stoichiometry was observed for the Arg-136 and Arg-321 mutants. The replacement of the positively charged arginine residues by an alanine might make available an additional Mg(II) binding site in the enzyme, but this higher Mg(II) binding did not compensate for the mutation in the enzyme. The Asp-111 mutant had only a slight reduction in the amount of bound Mg(II) when compared with the wild-type enzyme. The Glu-9 and Glu-115 mutants had the greatest reduction in the binding of Mg(II).

Change in Topoisomerase I Fluorescence from Mutation—The fluorescence spectra of the wild-type and mutant topoisomerase I mutants were first compared at room temperature (Fig. 8 and Table IV). The Glu-9 and Glu-115 mutants were found to have an ~30% drop in maximal fluorescence intensity, indicating a change in the protein conformation influencing the environment of the tryptophan residues in the enzyme. The decreases in fluorescence intensities were to a lesser extent for the Arg-136 and Arg-321 mutants (around 20%) while the Asp-111 and Asp-113 mutants had an ~25% drop in maximal fluorescence intensity. The fluorescence measurements were repeated at 42 °C. The wild-type enzyme fluorescence was not affected significantly by the temperature shift. In contrast, the fluorescence intensity of the Arg-321 mutant decreased by more than 40%, indicating lower stability. The Glu-9 mutant had a much smaller decrease in fluorescence intensity at the higher temperature (~14%) while the fluorescence intensities of the other mutants did not change significantly.

TABLE III

Mg(II) binding stoichiometry after equilibrium dialysis against buffer with 0.4 mM MgCl₂ for wild-type and mutant enzymes determined by ICP analysis

| Enzyme   | Mg(II)/molecule |
|----------|----------------|
| Wild-type| 2.15 ± 0.15    |
| E9A      | 1.34 ± 0.03    |
| D111A    | 1.90 ± 0.11    |
| D113A    | 1.85 ± 0.40    |
| E115A    | 1.36 ± 0.02    |
| R136A    | 2.50 ± 0.40    |
| R321A    | 2.90 ± 0.80    |

DISCUSSIONS

The site-directed mutagenesis study described here aimed at elucidating the function of several strictly conserved acidic or basic amino acid residues found at the proximity of the active site nucleophile Tyr-319. For Arg-136, mutation to alanine abolished relaxation activity totally. Mutations of the other residues produced enzymes with reduced but observable in vitro activities. Therefore, the strict conservation in evolution did not necessarily correlate with absolute requirement of the residue for in vitro activity. Nevertheless, all the mutants tested failed to complement E. coli AS17 for growth at 42 °C. This might at least partly be due to effect of the mutations on the stability and thus expression level of the enzyme in E. coli AS17. The Arg-136 mutant was the only one among the active site mutants tested here found to have an expression level in E. coli AS17 comparable with that of the wild-type enzyme. The
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The Arg-136, Arg-321, Asp-111, Asp-113, Glu-9, and Glu-115 mutants.

The spectra from \textit{E. coli} DNA topoisomerase I proteins at 25 °C. The values represent the average of three sets of measurements and are normalized relative to the maximum emission of wild-type topoisomerase I. Mutations of Glu-9 and Glu-115 reduced the Mg(II) binding stoichiometry significantly after equilibrium dialysis against buffer containing 0.4 mM MgCl\(_2\). This might have resulted from one of the multiple coordination sites for Mg(II) in the enzyme being removed or replaced by a ligand of lower affinity to Mg(II). The effects of the mutations of Glu-9, Asp-111, Asp-113, and Glu-115 to alanine on the relaxation activity were less severe than those observed for mutations of carboxylates proposed to be Mg(II) coordination sites involved in nucleotidyl transfer in other enzyme systems (11, 14, 30, 31). It is possible that for \textit{E. coli} DNA topoisomerase I, water or a DNA phosphate could substitute for the mutated carboxylate in Mg(II) coordination in the relaxation reaction to provide partial enzymatic activity, especially at Mg(II) concentration significantly above the minimal concentrations needed for relaxation activity. The effect of mutation of Asp-111 on the enzyme activity and Mg(II) binding was significantly smaller than mutations of the other carboxylates, so it is unlikely that Asp-111 is a catalytically important residue.

The Glu-9, Asp-113, Glu-115, and Arg-321 mutants also had reduced DNA binding activity. This could be due to the participation of the residue in direct protein-DNA interaction which is a plausible role for Arg-321. Alternatively, the reduced DNA binding could be due to the effect of the mutation on the protein folding. It also cannot be ruled out that the lower Mg(II) binding stoichiometries observed for the Glu-9 and Glu-115 mutants might be due to the effect of the mutations on the protein structure and not due to loss of a Mg(II) coordination site. A Mg(II) binding site distinct from the catalytic center has been proposed for the \textit{E. coli} DNA topoisomerase I (32). \textit{E. coli} DNA topoisomerase I may also have a second Mg(II) binding site away from the active site region that is required for relaxation activity because of its effect on the protein conformational changes that take place during the relaxation reaction cycle (28). The carboxylates at the active site may be conserved for their roles in protein structure instead of catalytic functions. The magnitude of the effect of a single mutation of these catalytically non-essential residues on the overall protein stability may depend on the \textit{E. coli} strain background, but nevertheless be of sufficient significance to account for the evolutionary conservation.

The steps of DNA cleavage and religation may or may not involve the same catalytic residues in the activation of nucleophiles as well as the stabilization of transition states and leaving groups. Presently there is a lack of data to address this question, and it remains unclear what these catalytic residues may be. Among the mutants tested, the Glu-9 mutant had the greatest loss of DNA cleavage activity with only a modest reduction in non-covalent DNA binding. Besides a possible role in binding Mg(II), this residue might be involved in the catalytic step of DNA cleavage. In contrast, the Arg-136 mutant had normal DNA cleavage activity but no relaxation activity. It might be needed in the DNA strand passage step since it could perform the inter-molecular rejoining of DNA.

There is also a lack of information on the orientation of the DNA substrate when it binds to the active site. Additional biochemical data and/or structural information of the enzyme-
DNA or enzyme-Mg(II) complexes would be needed to arrive at a more detailed mechanism of catalysis by the enzyme.

Acknowledgments—We thank Dr. James C. Wang for communications and discussions of results.

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