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Residues of *E. coli* topoisomerase I conserved for interaction with a specific cytosine base to facilitate DNA cleavage

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ABSTRACT

Bacterial and archaeal topoisomerase I display selectivity for a cytosine base 4 nt upstream from the DNA cleavage site. Recently, the solved crystal structure of *Escherichia coli* topoisomerase I covalently linked to a single-stranded oligonucleotide revealed that R169 and R173 interact with the cytosine base at the −4 position via hydrogen bonds while the phenol ring of Y177 wedges between the bases at the −4 and the −5 position. Substituting R169 to alanine changed the selectivity of the enzyme for the base at the −4 position from a cytosine to an adenine. The R173A mutant displayed similar sequence selectivity as the wild-type enzyme, but weaker cleavage and relaxation activity. Mutation of Y177 to serine or alanine rendered the enzyme inactive. Although mutation of each of these residues led to different outcomes, R169, R173 and Y177 work together to interact with a cytosine base at the −4 position to facilitate DNA cleavage. These strictly conserved residues might act after initial substrate binding as a Molecular Ruler to form a protein–DNA complex with the scissile phosphate positioned at the active site for optimal DNA cleavage by the tyrosine hydroxyl nucleophile to facilitate DNA cleavage in the reaction pathway.

INTRODUCTION

Maintenance of optimal DNA topology in cells relies on the action of ubiquitous enzymes called topoisomerases (1). Topoisomerases relieve superhelical stress by transiently cleaving, shuffling and rejoining DNA. This is achieved via a tyrosine residue (Y319 in *Escherichia coli* topoisomerase I) in the active site of topoisomerases. Topoisomerases are classified into two types based on the number of strands they cleave: Type I topoisomerases cleave a single strand of DNA while Type II topoisomerases cleave both strands of DNA. Type I and Type II topoisomerases are further subdivided into subclasses, each consisting of enzymes that are structurally and mechanistically similar (2). Although the distribution of topoisomerases varies from organism to organism depending on the functional roles to be fulfilled, the one unifying observation is the presence of at least one Type IA and one Type II topoisomerase in each organism (3). Type IB and Type IIA topoisomerases are well-established targets for anticancer and antibacterial drugs (4–8). The emergence of multi-drug resistance bacterial pathogens has led to urgency for the development of novel drug targets. Currently, there are no drugs available that target Type IA topoisomerases specifically. The presence of at least one Type IA topoisomerase in every bacterium makes this subclass of topoisomerases an attractive target for discovery of topoisomerase poison inhibitors that can lead to bacterial cell death through accumulation of the covalent DNA cleavage complex (9). In order to develop them as drug targets, a detailed understanding of the exact catalytic mechanism of action of Type IA topoisomerases is imperative.

DNA cleavage by topoisomerases does not occur at random sites. Previous studies have shown that *E. coli* topoisomerase I (EcTOP1), a Type IA topoisomerase, selectively cleaves DNA at sites with a cytosine base four bases 5′ to the DNA scissile phosphate (10,11). The selectively for this cytosine base has been noted for all other bacterial and archeal topoisomerase I as well as reverse gyrase enzymes characterized (12,13). There has been no indication of the biochemical basis for this conserved selectivity of the −4 cytosine base until the recent availability of the crystal structure of the EcTOP1 D111N mutant enzyme catalytic domain in covalent complex with a cleaved single-stranded 13 base oligonucleotide substrate (14). There is a cytosine base at the −4 position from the cleavage site in this oligonucleotide substrate. It was also observed in the crystal structure that there is a distinctive

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kink in the phosphate-deoxyribose chain of the oligonucleotide between −4 and −5 position bases. This kink disrupts the stacking of the bases of the oligonucleotide. Analysis of the residues around the −4C revealed two arginine residues R169 and R173 interacting with the cytosine base via hydrogen bonds (Supplementary Figure S1). Additionally, the phenol ring of a tyrosine residue Y177 wedges between the −4 and the −5 bases (14).

R169 and R173 are strictly conserved in bacterial and archael topoisomerase I and reverse gyrase enzymes, while a phenylalanine is seen in all reverse gyrase sequences at the position corresponding to Y177 (14). In contrast, topoisomerase III enzymes have different amino acid residues present at positions corresponding to R169, R173 and Y177 and are known to cleave DNA with different sequence selectivity (14). The specific selectivity of the cytosine base at the −4 position and the inferences from the crystal structure led to the hypothesis that R169, R173 and Y177 might play an important role in the catalytic mechanism of *E. coli* topoisomerase I. In order to analyze their role in DNA sequence selectivity and overall catalysis, residues R169, R173 and Y177 of EcTOP1 were substituted by site-directed mutagenesis. Biochemical analysis of the mutant enzymes showed that each of the mutations resulted in effects on DNA cleavage and relaxation activity distinct from each other. Based on the results reported here, it can be proposed that R169, R173 and Y177 function as a group to interact with the cytosine base at the −4 position and act as a Molecular Ruler to position the scissile phosphate at the active site for the DNA cleavage step of the catalytic cycle to proceed in the concerted reaction pathway following the initial binding of DNA substrate.

MATERIALS AND METHODS

Expression and purification of EcTOP1 wild-type and R169A, R173A and Y177S mutant enzymes

The expression and purification of EcTOP1 wild-type and mutant enzymes was performed as described earlier (15,16). Briefly, the EcTOP1 mutant clones were generated by site directed mutagenesis using the QuickChange procedures with Pfu Ultra II Fusion HS DNA polymerase (Stratagene). Plasmid pLIC-ETOP was used as a template. The ETOP coding sequence on pLIC-ETOP fused to a TEV protease cleavage 6× histidine tag is under the control of the T7 promoter (17). Plasmid pLIC-ETOP expressing either wild-type or mutant topoisomerase I was transformed into *E. coli* strain BL21AI (Invitrogen) with the T7 RNA polymerase coding sequence under the control of lacI and the BAD promoter. Recombinant protein expression was induced when $A_{600} = 0.4$ with the addition of 0.02% arabinose and 1 mM isopropyl 1-thio-ß-D-galactopyranoside. After induction for 3 h at 37°C, the cells were lysed by lysozyme treatment in lysis buffer (50 mM sodium phosphate, 0.3 M NaCl and 10 mM Imidazole, pH 8.0) and freeze thaw cycles (18). The soluble lysate containing the recombinant proteins was obtained by ultracentrifugation at 31 000 rpm for 2 h at 4°C. Initial purification was carried out by mixing the soluble lysate with Ni-NTA Agarose (Qiagen) in wash buffer (50 mM sodium phosphate, 0.3 M NaCl and 20 mM Imidazole, pH 8.0) before packing into a column. After extensive washing, the recombinant protein was eluted with elution buffer (50 mM sodium phosphate, 0.3 M NaCl, pH 8.0) containing 250 mM imidazole. Post elution, the N-terminal hexa-histidine tag was cleaved with tobacco etch virus protease and removed using Ni-NTA agarose again. Additional purification was achieved using a single-stranded DNA cellulose column (Sigma). The recombinant proteins were eluted using an increasing concentration gradient of KCl (18). The EcTOP1 R169A and Y177S mutant proteins were further purified using an S200 gel filtration column to remove nucleases co-eluted during the previous steps. The recombinant proteins were checked for purity by SDS–PAGE and found to be >99% homogeneous at the end of purification. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad) using BSA as standard.

Assay of relaxation activity

Wild-type and mutant EcTOP1 topoisomerases were serially diluted and assayed for relaxation activity in a standard reaction volume of 20 µl with 10 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml gelatin, 6 mM $MgCl_2$ and 0.2 µg of supercoiled pBAD/thio plasmid DNA (purified by CsCl density gradient centrifugation). After incubation at 37°C for 30 min, the reactions were stopped by addition of 4 µl of stop buffer [50 mM EDTA, 50% glycerol and 0.5% (v/v) bromophenol blue]. For relaxation assays performed to compare the rate of catalysis, 10 ng of wild-type EcTOP1 and 200 ng of mutant enzymes were incubated for 30, 60, 90, 120 and 180 min at 37°C before stopping the reactions. The DNA was electrophoresed in a 0.8% agarose gel with TAE buffer (40 mM Tris–acetate, pH 8.1, 2 mM EDTA). The gel was stained with ethidium bromide and photographed over UV light.

Cleavage of 556 base single-stranded DNA substrate

A 5′-^{32}P labeled single-stranded DNA substrate of 556 bases in length was generated by PCR using primers and plasmid pBAD/thio as template (12), followed by strand denaturation. The reverse primer was labeled with [γ-^{32}P] ATP in the presence of T4 polynucleotide kinase (New England BioLabs) prior to the PCR. The PCR products were purified using the DNA Clean and Concentrator Kit (Zymos) and eluted in 10 mM Tris–HCl, pH 8.0. Prior to the addition of EcTOP1 and mutant enzymes in the cleavage assay, the labeled DNA substrate was denatured to single strands by heating at 95°C for 10 min and rapidly cooled on ice. After incubation with 200 ng of enzyme 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. The reactions were incubated at 37°C for an additional minute. The reactions were stopped by adding an equal volume of gel loading solution (85% formamide, 25 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol). The samples were heated at 95°C for

5 min before electrophoresis on a 6% polyacrylamide sequencing gel. The 5′-end-labeled DNA cleavage products on the dried gel were visualized using a Storm 860 Phosphorimager. DNA sequencing reaction products were generated with the same 5′-end-labeled primer used to generate the substrate for the cleavage assay and by following the cycle sequencing procedures according to the manufacturer's instructions (SequiTherm DNA sequencing Kit, Epicentre). The sequencing reaction products were electrophoresed next to lanes containing cleavage products to identify the cleavage sites.

Cleavage and religation of 59 base hairpin oligonucleotide substrates

Oligonucleotide substrates Oligo A, Oligo C, Oligo G and Oligo T (Figure 3), forming a hairpin structure with 16 bp in the stem and 27 bases in the loop were labeled with [γ ^{32}P] ATP by T4 polynucleotide kinase (New England BioLabs). Wild-type EcTOP1 or the mutant enzymes were incubated with the labeled oligonucleotides at 37°C for 10 min in 10 mM Tris, pH 8.0 in the presence or absence of 0.5 mM $MgCl_2$. The reactions were stopped by adding an equal volume of stop solution (79% formamide, 0.2 M NaOH, 0.04% bromophenol blue). The samples were heated at 95°C for 5 min before electrophoresis in a 15% sequencing gel. The fraction of oligonucleotide cleaved by the enzymes was determined by analysis with the Phosphoimager Storm 860. The graphs for percent cleaved product represent the average and SD from at least three experiments performed separately.

For religation assays, post cleavage incubation, the cleavage reactions were cooled on ice for 10 min before the simultaneous addition of 0.5 mM $MgCl_2$ to initiate religation and 1 M NaCl to dissociate the enzyme from the religated DNA. The reactions were stopped by addition of equal volume of stop solution. The samples were heated at 95°C for 5 min before electrophoresis in a 15% sequencing gel. The fraction of oligonucleotide rejoined by the enzymes was determined by the decrease in the intensity of the cleaved product band using the Storm 860 Phosphorimager.

Anisotropy experiments to measure enzyme–DNA binding affinity

Enzyme–DNA binding affinity was measured using fluorescence anisotropy. Increasing concentrations (2–210 nM) of either wild-type EcTOP1 or the R169A, R173A and Y177S mutant enzymes were titrated into 5, 10, 15, 20, 25 and 30 nM solutions of the 59 base hairpin oligonucleotide substrates (Oligo C, Oligo A, Oligo G) (Figure 3) modified with 6-carboxyfluorescein at the 3′ end (synthesized by Biosearch Technologies). All measurements were performed at room temperature in 0.5 ml of binding buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA) (16). Excitation and emission wavelengths were 495 and 520 nm, respectively. The excitation and emission slits were set at 5 and 10 nm, respectively. Data was collected using the Advanced Reads program on a Varian Cary Eclipse fluorescence spectrophotometer. Control experiments were performed by titrating the fluorescently labeled oligonucleotide with volume of storage buffer corresponding to the enzyme additions. The increase in volume from addition of buffer or enzyme did not exceed 3.5% of the initial volume. Binding data was fit to the following equation for Binding Ligand Depletion using Anisotropy to determine Kd (dissociation constant) in GraphPad Prism software:

$$Y = (A_{\max}/z) \times (b - \mathrm{sqrt}(b \times b - 4 \times a \times c))/(2 \times a)$$

In this equation $a = 1$, $b = \mathrm{Kd} + X/n + z$, $c = X/n \times z$ and Kd is the dissociation constant, n is the number of titrant molecules/fluorescent molecule, z is the fluorescent substance concentration and $A_{\max}$ is the maximum relative anisotropy value.

Modeling of residues in wild-type or mutant EcTOP1 using PyMOL

Figures depicting the structures of residues R169, R173 and Y177 in wild-type EcTOP1 or their mutants were modeled in PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC) using the solved crystal structure of the 67-kD N terminal fragment of EcTOP1 D111N mutant enzyme in covalent complex with a 13 base single-stranded oligonucleotide (PDB ID 3PX7). Amino acid mutations were generated using the Mutagenesis wizard in PyMOL, whereas nucleic acid substitutions were generated using the winCOOT program (19).

RESULTS

Mutation of R169, R173 and Y177 residues led to loss in relaxation activity

The relaxation activity of wild-type EcTOP1 and the R169A, R173A and Y177S mutant enzymes was determined by incubating the purified enzymes with negatively supercoiled plasmid DNA. All three mutant enzymes were affected in their ability to relax DNA, when assayed for 30 min at 37°C (Figure 1A). The R169A mutant displayed approximately a 150-fold decrease in relaxation activity compared to wild-type EcTOP1. The R173A mutant displayed approximately a 100-fold decrease in relaxation activity compared to wild-type EcTOP1. The Y177S mutant showed a complete loss in relaxation activity (Figure 1A). The Y177A mutant enzyme was also purified and was found to also have null relaxation activity (data not shown). These results indicated that mutation of residues seen in the crystal structure to be interacting with the cytosine base in the −4 position could affect the functional activity of the enzyme by disrupting the catalytic cycle.

In order to determine if the effect observed in the relaxation assay was due to a decreased rate of catalysis, the wild-type EcTOP1 and the R169A, R173A and Y177S mutant enzymes were incubated with the supercoiled plasmid DNA substrate for longer time periods. The R169A and R173A mutant enzymes formed slightly more relaxed DNA products after incubation for over 120 min (Figure 1B). However, the Y177S mutant was

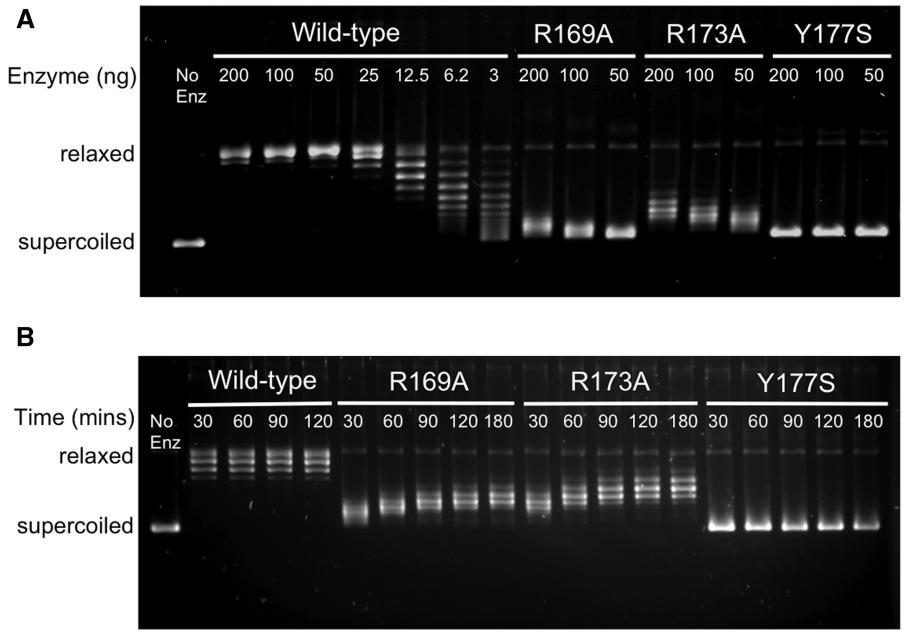

Figure 1. Relaxation assay comparing wild-type EcTOP1 activity against R169A, R173A and Y177S mutant enzymes. (**A**) Wild-type EcTOP1 and R169A, R173A and Y177S mutant enzymes were serially diluted and added to a reaction mixture containing 0.2 μg of negatively supercoiled plasmid DNA. Incubation was at 37°C for 30 min. (**B**) 10 ng of wild-type EcTOP1 and 200 ng R169A, R173A and Y177S mutant enzymes were incubated with 0.2 μg of negatively supercoiled plasmid DNA at 37°C for the indicated time periods. No enz, no enzyme.

incapable of relaxing DNA even with extended incubation times (Figure 1B). The decreased rate of DNA relaxation by the R169A and R173A mutants suggested that mutation of these residues affected the rate of catalysis of the enzyme.

Effect of EcTOP1 R169A, R173A and Y177S mutations on DNA cleavage activity

The relaxation assay results suggested that mutation of residues R169, R173 and Y177 led to a disruption in the catalytic cycle of the enzyme. Hence, cleavage, religation and binding assays were performed to determine which step of the catalytic cycle was disrupted.

Since the three residues being studied were observed in the crystal structure to interact with the cytosine base at the −4 position, the disruption in the catalytic cycle observed may be due to an inability of the mutant enzymes to recognize the cytosine base. To check if mutation of the residues led to a change in selectivity of cleavage sites, wild-type EcTOP1 and the R169A, R173A and Y177S mutant enzymes were incubated with a 556 base single-stranded DNA substrate labeled with ^{32}P at the 5′ end. Sequencing reactions with the labeled substrate as template were run along with the cleavage reactions to map the base at the −4 position of cleaved site. The results showed that wild-type EcTOP1 cleaved the labeled substrate at multiple sites, all of which have a cytosine base at the −4 position (Figure 2). The R173A mutant enzyme displayed a cleavage pattern similar to wild-type, however the level of cleaved DNA formed was significantly lower. The Y177S mutant enzyme was incapable of DNA cleavage. However, a noticibly different result was observed when the labeled substrate was cleaved by the R169A mutant enzyme. This enzyme cleaved DNA at sites distinct from the wild-type EcTOP1 DNA cleavage sites (Figure 2). When the cleavage sites were mapped on the sequence of the substrate, it was observed that all the sites cleaved by the R169A mutant had an adenine base at the −4 position. The result observed with the mutation of the R169 residue demonstrated a change in the sequence selectivity of the mutant enzyme from a cytosine to an adenine base at the −4 position.

Designing oligonucleotide substrates for specific cleavage, religation and binding experiments

Type IA topoisomerases require a single-stranded substrate in order to relieve topological stress. Cleavage assays using long DNA substrates reveal multiple cleaved products. Shorter oligonucleotides can be designed which are cleaved specifically at one site as shown for Oligo C (Figure 3), a 59 base oligonucleotide which forms a hairpin structure. This structure provides the enzyme with both single- and double-stranded regions mimicking the negatively supercoiled duplex DNA substrates for topoisomerase I *in vivo*. Oligo C has a preferred cleavage site in the single-stranded region recognized by EcTOP1 (Figure 3, arrow). DNA cleavage experiments using Oligo C labeled with ^{32}P at the 5′ end confirmed that wild-type EcTOP1 recognizes the cytosine base at the 22nd position and cleaves the substrate at one site 4 bases from this cytosine (Figure 4A). Using Oligo C as a template, three additional oligonucleotides were designed, each of them differing from Oligo C in the base at position 22 from the 5′ end. These oligonucleotides were named Oligo A, Oligo G and Oligo T, corresponding to the base at the 22nd position from the 5′ end (Figure 3).

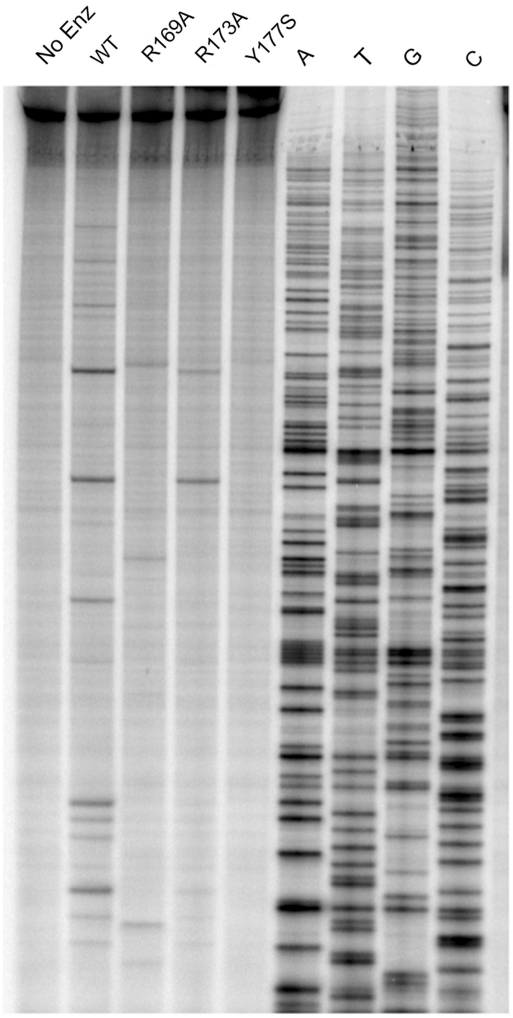

Figure 2. DNA cleavage by wild-type EcTOP1 and R169A, R173A and Y177S mutant enzymes. A 556 base DNA substrate labeled with ^{32}P at the 5′ end was incubated with 200 ng of wild-type EcTOP1 (WT), and the R169A, R173A and Y177S mutant enzymes at 37°C for 30 min. DNA cleavage products were analyzed by electrophoresis in a 6% sequencing gel. Lanes A, T, G and C containing the sequencing reactions with the corresponding nucleotide termination mixtures were electrophoresed along with the cleavage reactions. No enz, no enzyme.

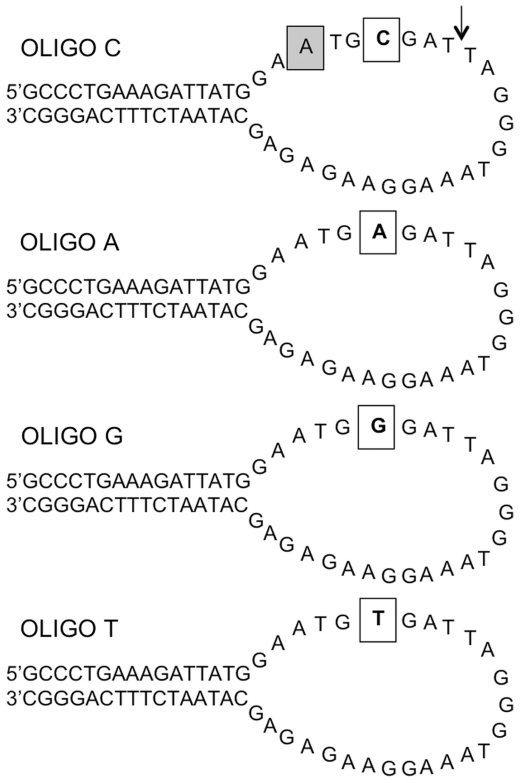

Figure 3. Hairpin structures and sequences of the four oligonucleotides designed. Four 59 base oligonucleotides forming a hairpin structure, designed to check for cleavage by wild-type EcTOP1 and R169A, R173A and Y177S mutant enzymes are shown. The four oligonucleotides differ in the base at position 22 from the 5′ end (base shown in clear box). The arrow indicates the site on Oligo C cleaved by wild-type EcTOP1. R169A cleaves Oligo C at an additional site (Figure 4), which has an adenine base at the −4 position (gray box). The folding of the oligonucleotides was checked using the MFold program (20).

These oligonucleotide substrates were used in experiments to study DNA cleavage, religation and binding by the R169A, R173A and Y177S mutant enzymes.

Cleavage of Oligo C, Oligo A, Oligo G and Oligo T by EcTOP1 wild-type and R169A, R173A and Y177S mutant enzymes

In order to confirm the change in sequence selectivity by the R169A mutant enzyme, Oligo C and Oligo A were labeled with ^{32}P at the 5′ end and incubated with wild-type EcTOP1 and the R169A mutant enzymes. The labeled oligonucleotides were also incubated with R173A and Y177S to determine if these two mutant enzymes were capable of DNA cleavage. Additionally, Oligo G and Oligo T were also labeled and incubated with the enzymes to see if cleavage occurs.

Wild-type EcTOP1 cleaves Oligo C very strongly at one site, while the R169A mutant could cleave the labeled oligonucleotide weakly at two sites (Figure 4A) quantitated in Figure 4B as R169A-1 and R169A-2. Nuclease digestion of labeled Oligo C was carried out to determine that the base at the −4 position for R169A-2 was an adenine (Figure 3, Oligo C, gray box). R173A cleaved Oligo C at the same single site as wild-type EcTOP1 but the level of cleaved product was lower (Figure 4B). Y177S was incapable of cleaving Oligo C. R169A was the only enzyme which could cleave Oligo A, even though the level of cleaved product was 2- to 3-fold lower when compared to Oligo C cleaved product by wild-type EcTOP1 (Figure 4). Wild-type EcTOP1, R173A and Y177S did not cleave Oligo A at all. None of the enzymes could cleave Oligo G. Oligo T was cleaved by wild-type EcTOP1, R169A and R173A. However, the cleavage activities were very weak (Figure 4A). The results observed with this experiment are in agreement with the results seen in Figure 2 with the 556 base long single-stranded DNA substrate. Mutation of R169 to alanine changes the sequence selectivity of the enzyme from a cytosine base to an adenine base four bases 5′ to the cleavage site. In addition, it was also confirmed that

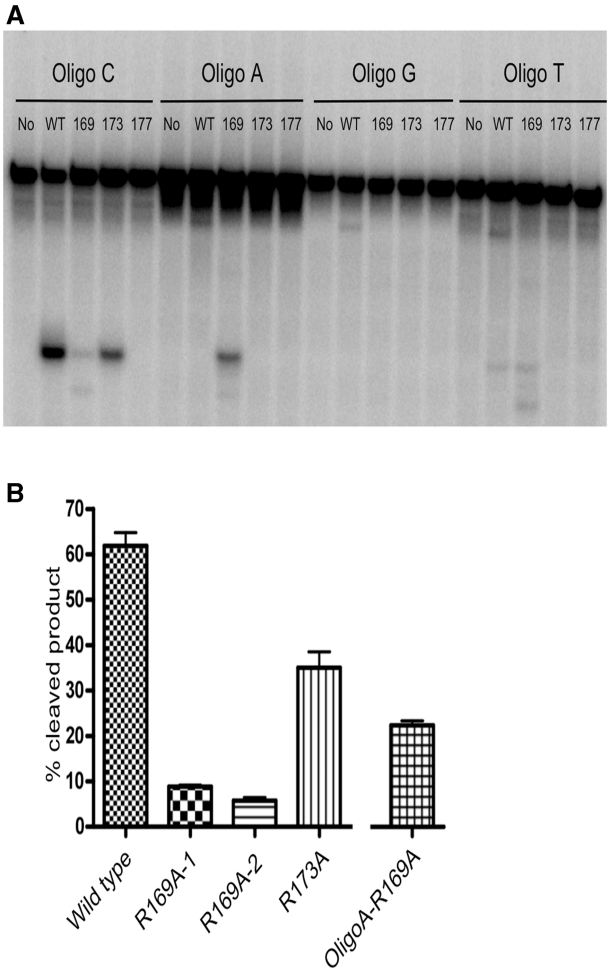

Figure 4. Cleavage of Oligo C, Oligo A, Oligo G and Oligo T by EcTOP1 wild-type, and R169A, R173A and Y177S mutant enzymes. (**A**) Oligo C, Oligo A, Oligo G and Oligo T labeled with ^{32}P at the 5′ end were incubated with wild-type EcTOP1 and R169A, R173A and Y177S mutant enzymes at 37°C for 10 min. DNA cleavage products were analyzed by electrophoresis in a 15% sequencing gel. No, no enzyme; WT, wild-type; 169, R169A; 173, R173A; 77, Y177S. (**B**) The percent of Oligo C cleaved product by wild-type EcTOP1, R169A and R173A enzymes and percent of Oligo A cleaved product by R169A mutant enzyme was quantitated with spot densitometry analysis. R169A-1 and R169A-2 represent the two cleaved products of Oligo C from top to bottom observed in part A. The average and SD from at least three separate experiments is shown.

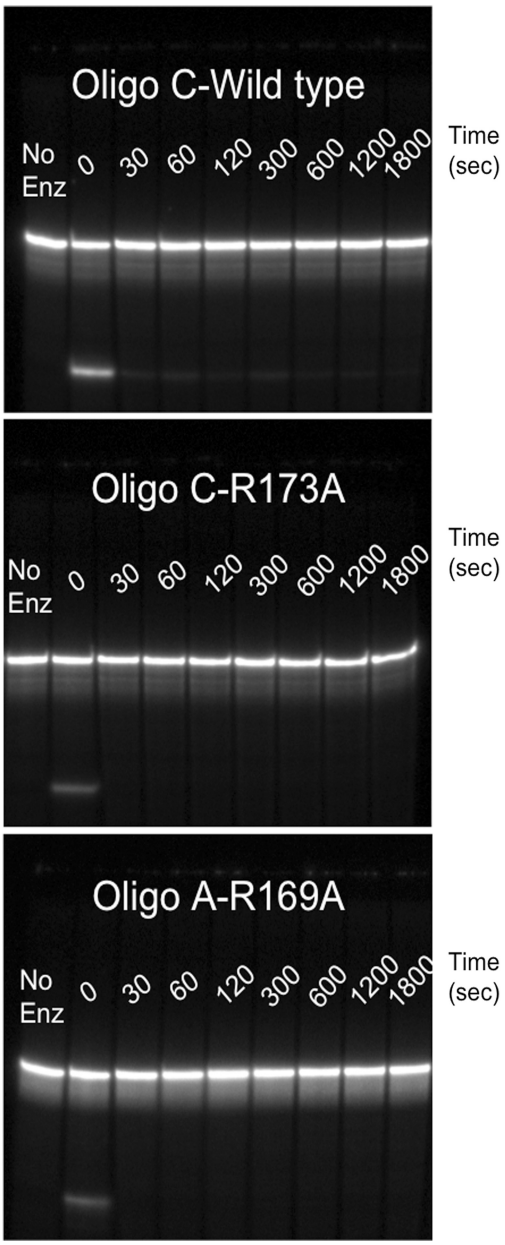

Figure 5. Oligo C religation by wild-type EcTOP1 and R173A; Oligo A religation by R169A. Topoisomerase cleavage reactions were incubated with 0.5 mM $MgCl_2$ and 1 M NaCl to dissociate the enzyme from DNA after religation of the covalent cleaved complex. Aliquots of reactions were stopped at the indicated time points and electrophoresed in a 15% sequencing gel. The levels of DNA substrate and cleaved products were analyzed by Phosphorimager. No enz, no enzyme.

although less active, R173A retained the wild-type EcTOP1 cleavage sequence selectivity.

DNA religation by wild-type EcTOP1, R169A and R173A

The ability of these mutant enzymes to religate DNA was also compared to wild-type EcTOP1. The R169A enzyme has a preference for an adenine at the −4 position for DNA cleavage and since it cleaved Oligo A better than Oligo C, Oligo A was used to assay the DNA religation activity of R169A enzyme. Oligo C was used to assay the religation activity of wild-type EcTOP1 and R173A mutant enzyme.

As seen in Figure 5, wild-type EcTOP1 efficiently religated Oligo C post cleavage. Although cleavage of Oligo C by R173A was weaker than wild-type, the mutant was extremely efficient at religating Oligo C post cleavage. Similarly, R169A demonstrated efficient religation of Oligo A post cleavage. These results suggest that mutation of the R169 and R173 residues to alanine does not affect the ability of the enzyme to religate DNA despite weaker/differential cleavage activity. Since the Y177S mutant enzyme demonstrated a complete loss in cleavage activity, the religation activity of this mutant could not be assessed.

Binding of wild-type EcTOP1 and the R169A, R173A and Y177S mutant enzymes to the hairpin oligonucleotides

The R169A and R173A mutant enzymes demonstrated efficient religation activity, while their ability to cleave DNA was compromised when compared to wild-type. The Y177S mutant was unable to cleave DNA. These results pose an important question: is the decreased/differential DNA cleavage activity observed by the mutant enzymes due to decreased affinity to the DNA substrate or due to inefficient positioning of the DNA–protein interactions for the cleavage step to take place? The importance of R169, R173 and Y177 residues in selectivity of the cytosine base at the −4 position encompasses the possibility of accurately positioning the DNA substrate relative to the catalytic residues in the active site such that the tyrosine side chain responsible for DNA cleavage has access to the scissile DNA phosphate. Hence, to determine the effect of the selectivity of the −4 cytosine base on initial DNA binding, fluorescence anisotropy binding assays were performed. Oligo C, Oligo A and Oligo G were labeled with 6-carboxyfluorescein at the 3′ end and six different concentrations of the labeled oligonucleotides were titrated with increasing concentrations of wild-type EcTOP1, R169A, R173A and Y177S mutant enzymes. Labeled Oligo C, which has a cytosine base at the −4 position, was titrated with wild-type EcTOP1, R169A, R173A and Y177S mutant enzymes. Oligo A, which has an adenine base at the −4 position, was cleaved only by the R169A mutant enzyme (Figure 4A). Hence, titrations for labeled Oligo A were performed with the R169A mutant enzyme and wild-type EcTOP1 for comparison. Since no cleavage was observed for Oligo G by either wild-type or the mutant enzymes, it would be interesting to see if the enzymes are still capable of binding to this oligonucleotide. Hence labeled Oligo G was titrated with wild-type EcTOP1 and the R169A, R173A and Y177S mutant enzymes. The change in anisotropy upon increasing concentration of enzyme was measured using a fluorescence spectrophotometer and the data were fit in GraphPad Prism using the equation for Binding Ligand Depletion using Anisotropy. The Kd values obtained by fitting the data are shown in Table 1.

Previous experiments performed with an EcTOP1 mutant enzyme with a substitution of an arginine residue R195 to alanine demonstrated the importance of this residue in DNA phosphate binding (21). Hence, EcTOP1 R195A mutant enzyme was used as a positive control to check for the validity of using fluorescence anisotropy as a technique to measure DNA binding affinity. The Kd value obtained with the EcTOP1 R195A mutant enzyme was ~3.5-fold higher than wild-type (data not shown), similar to the estimation previously made from the results of gel shift assay (22).

As expected, wild-type EcTOP1 binds to Oligo C strongly (Kd = 0.31 nM) (Table 1). R173A and Y177S enzymes bind to Oligo C with weaker affinity than wild-type EcTOP1; but the Kd values still indicate strong binding (<2-fold reduction when compared to wild-type) (Table 1).

Table 1. Dissociation constants (Kd) obtained for binding of wild-type EcTOP1, R169A, R173A and Y177S mutant enzymes to 3′-labeled Oligo C, Oligo A and Oligo G

3′-labeled oligonucleotide	Enzyme	Kd (nM)
Oligo C	Wild-type	0.31 ± 0.05
	R169A	0.86 ± 0.15
	R173A	0.57 ± 0.14
	Y177S	0.48 ± 0.02
Oligo A	Wild-type	0.40 ± 0.12
	R169A	0.55 ± 0.12
Oligo G	Wild-type	0.50 ± 0.02
	R169A	0.97 ± 0.19
	R173A	0.61 ± 0.09
	Y177S	0.46 ± 0.08

Kd values of the wild-type and mutant enzymes binding to the oligonucleotide substrates were obtained by fitting the fluorescence anisotropy binding data to an equation measuring binding ligand depletion using anisotropy in GraphPad Prism. Values represent average and SD from three separate experiments.

Wild-type EcTOP1 is incapable of cleaving Oligo A (Figure 4); however the binding affinity of the wild-type enzyme to Oligo A is comparable to R169A (Table 1), which is the only enzyme which cleaves Oligo A. This result suggests that recognition of the base at the −4 position for DNA cleavage may not be indicated by the substrate binding affinity. This is confirmed by the comparable Kd value observed for wild-type EcTOP1 binding to Oligo G (Table 1). As observed in the oligonucleotide cleavage assay, neither the wild-type EcTOP1 nor the mutant enzymes cleave Oligo G; but the binding affinity of wild-type EcTOP1 to Oligo G is comparable to the binding affinity of wild-type EcTOP1 to Oligo C.

It was interesting to see that the binding affinity of R169A to Oligo A was stronger than its binding affinity to Oligo C or Oligo G (Table 1). The Kd value for R169A binding to Oligo C was 0.86 nM, which is ~2.7-fold weaker than wild-type-Oligo C binding. The R169A binding data for all three oligonucleotides tested indicate that along with the change in cleavage selectivity, mutation of this residue does affect the substrate binding affinity of the enzyme to some extent.

The Kd values obtained for the R173A mutant enzyme are more similar to the affinities for wild-type EcTOP1, indicating that this mutation does not affect the substrate binding affinity of the enzyme as significantly as R169A. A similar result was observed for the Y177S mutant enzyme; the substrate binding affinities of Y177S are comparable to wild-type. Hence, the total lack of DNA cleavage or relaxation activity by the Y177S mutant enzyme is not due to loss of its binding affinity for the DNA substrate. The mutation is also unlikely to have resulted in a large change in protein folding.

DISCUSSION

Bacterial and archaeal topoisomerase I as well as reverse gyrase display a sequence selectivity of a cytosine base 4 bases 5′ from the DNA cleavage site. The biochemical basis for this selectivity was previously unknown.

Recently, the solved crystal structure of EcTOP1 D111N mutant enzyme in covalent complex with a cleaved single-stranded DNA shed light upon the residues which interact with the cytosine at the −4 position relative to the cleavage site (14). These residues R169, R173 and Y177 were mutated in order to analyze their biochemical role in the recognition and selectivity of the cytosine at the −4 position. Each of the mutant enzymes demonstrated biochemical properties distinct from each other.

Mutation of only one of the residues, R169 to alanine, resulted in a change in the sequence selectivity of EcTOP1. R169A mutant enzyme displayed higher cleavage activity when the base at the −4 position is an adenine compared to a cytosine (Figure 4). Once cleaved, the R169A mutant was efficient at religating cleaved DNA (Figure 5). Although R169A cleaved DNA with higher efficiency when the base at the −4 position is an adenine (Oligo A, Figure 4) as opposed to a cytosine (Oligo C, Figure 4), the cleavage activity of R169A was lower than wild-type EcTOP1 acting on Oligo C (Figure 4B). The mutant was found to have a 150-fold decrease in DNA relaxation activity compared to wild-type (Figure 1A). The decreased relaxation activity could be explained in part also by the lower binding affinity of R169A to DNA substrate when compared to wild-type EcTOP1 (Table 1). The solved crystal structure of EcTOP1 D111N mutant enzyme in covalent complex with DNA determined that the pocket created at the −4 position is capable of only accomodating a cytosine. R169 plays an important role in sterically restricting the choice of the base at the −4 position (Figure 6A). Mutation of R169 to alanine creates a larger pocket (Figure 6B, arrow) which could accommodate and interact with a larger base like adenine (Figure 6C). Measurement of the distance between the alanine residue and the adenine base in the model indicated the possibility of van der Waals interaction (data not shown). The extra space created due to the larger pocket in this R169A mutant would not allow tight interactions between the residues and the −4 base if the base is a pyrimidine. Although sterically the pocket could potentially fit a guanine base, the data suggests that the R169A mutant might be incapable of interacting non-covalently with a guanine as the −4 base, demonstrated by the inability of the R169A mutant to cleave Oligo G (Figure 4). Modelling studies indicated a steric interference in interaction with R173 when the base at the −4 position is a guanine (data not shown).

Mutation of R173 to an alanine retained the selectivity of a cytosine at the −4 position. The R173A mutant cleaved DNA with sequence selectivity similar to wild-type EcTOP1; however, the level of cleaved product observed was lower (Figures 2 and 4B). Mutation of residue R173 did not affect the binding affinity of the enzyme significantly (Table 1) and also did not change the ability of the enzyme to religate cleaved DNA (Figure 5). Since binding and religation were unaffected due to the mutation, the decrease in the cleavage activity could account for the lower rate of relaxation by R173A when compared to wild-type EcTOP1 (Figure 1A). Modeling of the R173 residue around the cytosine at the −4 position showed that this residue does not contribute sterically to the selection of the cytosine base, but is important for non-covalently interacting with the base at the −4 position (Figure 7A). Mutation of R173 to alanine would interfere with the ability of the residue to hydrogen bond with the cytosine base at the −4 position (Figure 7A) which then might affect the cleavage activity of the mutant enzyme compared to wild-type EcTOP1.

Mutation of the Y177 residue to a serine renders the enzyme completely inactive. The Y177S mutant is not affected in non-covalent binding affinity to DNA substrate (Table 1), but it is incapable of DNA relaxation and cleavage (Figures 1A, 2 and 4A). These results suggest a vital catalytic role for residue Y177 after the initial binding of DNA substrate, even though it is distal to the active site Y319 and the scissile phosphate. As observed in the crystal structure, the side chain of residue Y177 forms a wedge between the bases at the −4 and the −5 position. The phenol ring of Y177 stacks parallel to the guanine base at the −5 position and perpendicular to the cytosine base at the −4 position (Figure 7B). Y177 interacts with the bases at the −4 and the −5 position via π–π interactions. Y177 is strictly conserved in bacterial topoisomerase I and the corresponding residue in archaeal reverse gyrase is also strictly conserved as the aromatic amino acid phenylalanine. Mutation of Y177 to a serine residue would abolish the interaction of this residue with the bases at the −4 and the −5 position (Figure 7B). The major impact of mutating Y177 to a serine, however, would be on the wedging action of this residue. The selectivity of the cytosine at the −4 position might be an important step in the conformational change of the enzyme–DNA complex after initial binding so that the active site tyrosine could be precisely positioned for cleaving the single strand. The wedge created by Y177 could be a mechanism to latch on to the DNA strand upon appropriate recognition of the cytosine base, so that the active site tyrosine hydroxyl nucleophile has access to the scissile phosphate. This mechanism of recognizing the substrate at a site distant from the cleavage site is known as Molecular Ruler Mechanism. An example of the Molecular Ruler mechanism has been demonstrated in Type I restriction modification enzymes. These enzymes consist of three subunits; restriction, methylation and specificity subunits. The specificity subunit binds to two DNA sequences spaced 5–8 bp apart and determines whether the enzyme would act as an endonuclease or methylase. These enzymes differ from Type II and Type III restriction modification enzymes, because cleavage or methylation by Type I enzymes occur at the site distinct from the recognition site (23). The crystal structure of the specificity subunit of a Type I R-M enzyme from *Methanococcus jannaschii* revealed the presence of two highly conserved regions which form a coiled coil that separates the two DNA binding domains, thereby acting as a Molecular Ruler for the spacing between the two sequences bound by the DNA binding domain. Their results also suggested bending of the target DNA for exposure of the adenine bases to be methylated by the methylation subunit of the enzyme (24). Molecular rulers are very common among

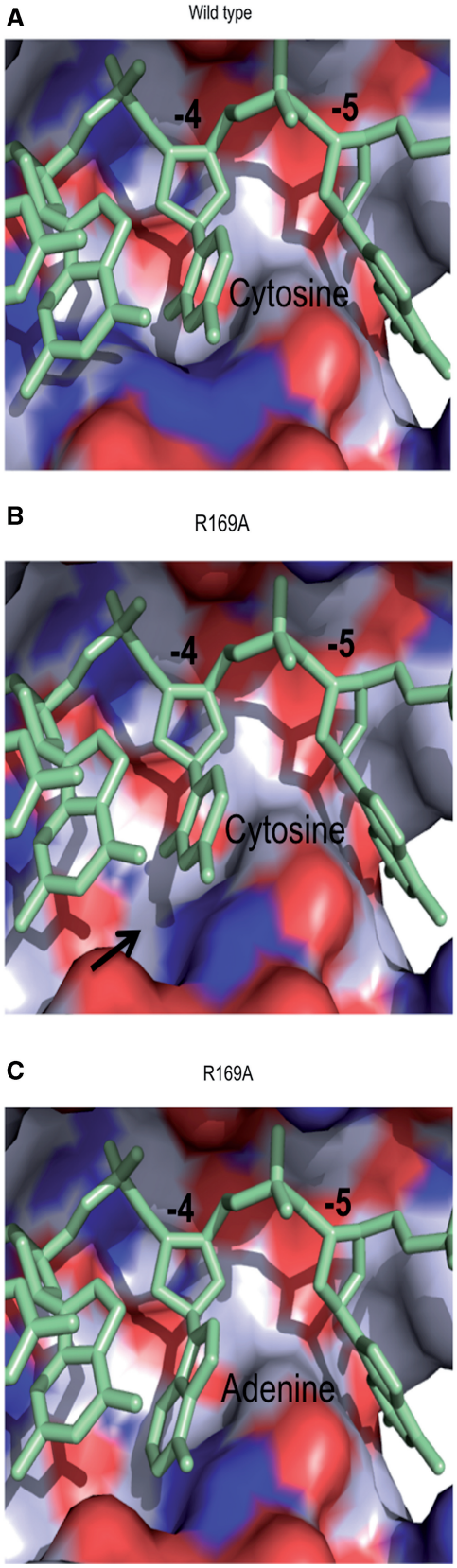

Figure 6. Modeling of the interaction of R169 and its alanine substitution mutant with the base at the −4 position. (**A**) Figure shows the surface representation of the wild-type enzyme bound to a single-stranded oligonucleotide with a cytosine base at the −4 position. R169 sterically restricts the choice of the base at the −4 position.

protein–protein interactions as well. An example is the cleavage of the thyrotropin receptor into two subunits by what is presumed to be a matrix metalloprotease. Cleavage of the receptor by the enzyme occurs at the fixed distance from the protease attachment site (25). Deletion of residues on the receptor at the protease attachment site decreased the cleavage activity and shifted the cleavage site to an upstream region.

Hence, recognition and selectivity of the cytosine base at the −4 position could be due to R169, R173 and Y177 residues functioning as a Molecular Ruler. These residues are therefore strictly conserved only in Type IA topoisomerases that maintain this cleavage sequence selectivity. The combined action of these three residues might be important in inducing conformational change in the enzyme–DNA complex after initial DNA binding to appropriately position or activate the active site tyrosine for cleavage of DNA. Initial DNA binding is likely to occur via residues recognizing DNA ribose-phosphate as seen in previous crystal structures of non-covalent complex between DNA and Type IA topoisomerases (26). These include residues E115, R168, D172, S192, R195 and Q197 of EcTOP1 that have been shown in previous mutagenesis studies to be important for DNA relaxation activity (14,21). Unlike R169, R173 and Y177 responsible for interaction with the cytosine base, these residues that interact with the DNA backbone are conserved among all Type IA topoisomerases regardless of their cleavage sequence selectivity (27).

Based on the results obtained in this study along with previous studies of EcTOP1, we propose the following sequence of events: (a) EcTOP1 binds to DNA via residues that recognize the DNA ribose-phosphate backbone leading to initial complex formation. (b) After initial complex formation, residue R169 recognizes a cytosine base at the −4 position relative to the site that needs to be cleaved. (c) Once recognized, non-covalent interactions of residues R169, R173 and Y177 with the cytosine base at the −4 position and the wedging action of the Y177 residue lead to a change in conformation of the enzyme–DNA complex so that the active site tyrosine is positioned or activated for efficient DNA cleavage, thereby acting as a Molecular Ruler.

The crystal structure of the D111N mutant in covalent complex in single-stranded DNA oligonucleotide substrate revealed a kink in the DNA phosphate-deoxyribose chain of the oligonucleotide between the −4 and −5 bases which disrupts the stacking of the bases (14). A recent study reported a novel, previously uncharacterized sequence of events during DNA cleavage by human topoisomerase II wherein post non-specific DNA binding, the

Figure 6. Continued
(**B**) Mutating R169 to alanine creates a larger pocket in the region surrounding the base at the −4 position (arrow). (**C**) This larger pocket created due to the R169A mutant could accommodate a larger base like adenine. The figures were made by mutating the R169 residue in PyMOL using the solved crystal structure of D111N EcTOP1 mutant enzyme in covalent complex with single-stranded DNA (PDB ID 3PX7). Substitution of the cytosine base to adenine was performed using winCOOT (19).

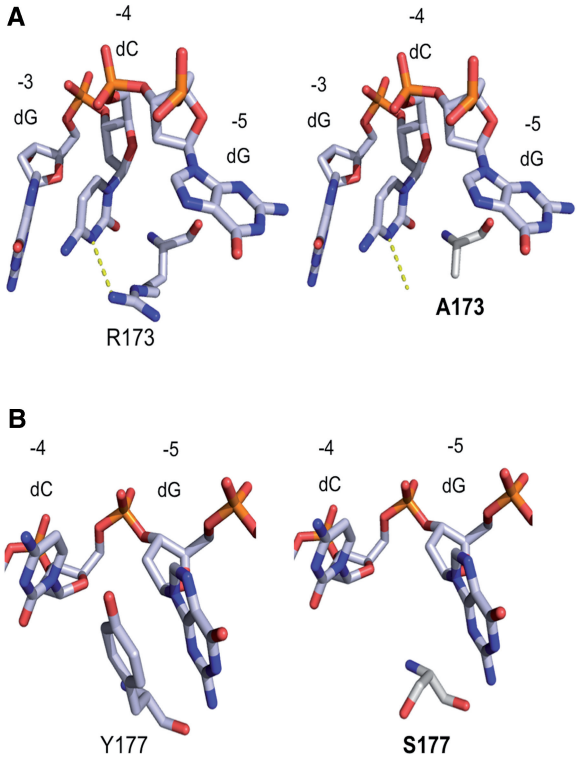

Figure 7. Modeling of the interaction of R173 and Y177 and their substitution mutants with the cytosine base at the −4 position. (**A**) Figure depicts the effect of mutating R173 to alanine (A173, red) on its interaction with the cytosine base. The hydrogen bond formed is depicted by the yellow dashed line. (**B**) Figure depicts the effect of mutating Y177 to serine (S177, red) on its interaction with the cytosine base. S177 would be incapable of forming the wedge. The figures were made by mutating the R173 and Y177 residues in PyMOL starting from the solved crystal structure of D111N EcTOP1 mutant enzyme in covalent complex with single-stranded DNA (PDB ID 3PX7).

enzyme induces a sharp bend in DNA in a sequence specific manner prior to DNA cleavage (28). Their data suggested a tight coordination between DNA bending and DNA cleavage at a selected site by topoisomerase II. It is possible that the kink observed in the sugar-phosphate backbone in the crystal structure of EcTOP1 D111N bound to single-stranded DNA could be employed in co-ordination with the recognition of the cytosine base at the −4 position to facilitate DNA cleavage. Further studies should be carried out to elucidate further details of this mechanism in bacterial topoisomerase I action.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figure 1.

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