Identification of Active Site Residues in *Escherichia coli* DNA Topoisomerase I*

(Sue-Jane Chen and James C. Wang‡)

From the Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

Alanine substitution mutagenesis of *Escherichia coli* DNA topoisomerase I, a member of the type IA subfamily of DNA topoisomerases, was carried out to identify amino acid side chains that are involved in transesterification between DNA and the active site tyrosine Tyr-319 of the enzyme. Twelve polar residues that are highly conserved among the type IA enzymes, Glu-9, His-33, Asp-111, Gln-115, Glu-309, Glu-313, Thr-318, Arg-321, Thr-322, Asp-323, His-365, and Thr-496, were selected for alanine substitution. Each of the mutant enzymes was overexpressed, purified, and characterized. Surprisingly, only substitution at Glu-9 and Arg-321 was found to reduce the DNA relaxation activity of the enzyme to an insignificant level. The R321A mutant enzyme, but not the E9A mutant enzyme, was found to retain a reduced level of DNA cleavage activity. Two additional mutant enzymes R321K and E9Q were also constructed and purified. Replacing Arg-321 by lysine has little effect on enzymatic activities; replacing Glu-9 by glutamine greatly reduces the supercoil removal activity but not the DNA cleavage and rejoining activities. From these results and the locations of the amino acids in the crystal structure of the enzyme, it appears that Glu-9 has a critical role in DNA breakage and rejoining, probably through its interaction with the 3′ deoxyribosyl oxygen. The positively charged Arg-321 may also participate in these reactions by interacting with the scissile DNA phosphate as a monodentate. Because of the strict conservation of these residues, the findings for the *E. coli* enzyme are likely to apply to all type IA DNA topoisomerases.

DNA topoisomerases are enzymes that participate in nearly all cellular transactions of DNA, including replication, transcription, and chromosome condensation (for reviews see Ref. 1 and references therein). There are two types of DNA topoisomerases: the type I enzymes catalyze the transport of individual DNA strands through one another and the type II enzymes catalyze the interpenetration of double-stranded DNA segments. By doing so, the DNA topoisomerases alleviate the topological problems encountered by intracellular DNA.

The transport of DNA strands through one another requires the transient breakage of one of the encountering pair, and all DNA topoisomerases catalyze this reaction through the formation of covalent enzyme-DNA intermediates; the phenolic oxygen of an enzyme tyrosyl residue undergoes nucleophilic attack of a DNA phosphorous to break a DNA phosphodiester bond and form a phosphotyrosine link (2, 3). Following DNA strand breakage and passage, the deoxyribosyl hydroxyl formed during DNA breakage acts as the nucleophile to break the phosphotyrosine link and rejoin the DNA strand.

By analogy to the cleavage of DNA by nucleases (4) and from studies of the pH dependencies of the reaction steps catalyzed by vaccinia virus topoisomerase (5, 6), it was suggested that transesterification mediated by the DNA topoisomerases might involve general acid-base catalysis. In the formation of the enzyme-DNA covalent adduct, a general base in the enzyme might assist in the removal of the hydroxyl proton of the active site tyrosine, and a separate general acid in the enzyme might assist in the protonation of the departing deoxyribosyl oxygen. The DNA rejoining reaction after strand passage could be the exact microscopic reversal of the DNA breakage reaction (5–7), but there has been no definitive experimental test of this conjecture.

In this work, we report a mutational analysis of *Escherichia coli* DNA topoisomerase I for the identification of amino acid side chains that might be directly involved in transesterification between Tyr-319 of the enzyme and the DNA scissile phosphorus (4). The *E. coli* enzyme is representative of a subfamily of type I DNA topoisomerases, the type IA enzymes, from a diverse collection of organisms including bacteria, eukaryotes, and archaea (1, 8). Alignment of amino acid sequences of these enzymes showed a large number of highly conserved amino acid residues (9). This subfamily of enzymes is very different, however, from the type IB DNA topoisomerases, whose members include eukaryotic DNA topoisomerase I and vaccinia virus topoisomerase, both in amino acid sequences and reaction mechanisms (1). Several studies on the identification of active site residues in vaccinia virus DNA topoisomerase have been reported recently (10–13).

*E. coli* DNA topoisomerase I is encoded by the *topA* gene comprised of 865 codons (14). For convenience, the positions of all amino acid residues in the 97-kDa single polypeptide protein are referred to by their codon numbers, even though the N-terminal methionine is removed post-translation (14). The three-dimensional structure of a 67-kDa fragment of the enzyme has been determined by x-ray crystallography (15), and that of a C-terminal fragment comprised of amino acids 745–865 has been determined by nuclear magnetic resonance (16). The latter fragment is dispensable for catalytic activity but appears to participate in substrate binding (17, 18). The polypeptide bridging these two fragments contains three motifs with four cysteines in each. These tetracysteine motifs are most likely the binding sites of three Zn(II) ions (19).

The 67-kDa N-terminal fragment is capable of covalent adduct formation with single-stranded DNA (15). Therefore, it is most likely to contain all residues that are essential for covalent catalysis. In the crystal structure of this fragment, the polypeptide is folded into four distinct domains (15). The frag-
ment can be viewed to comprise a "base" formed by domains I and IV and a "lid" formed by domains II and III (see Fig. 1A). In the crystal, the base and the lid are touching on one side through contacts between domain III in the lid and domains I and IV in the base and are linked on the other side by a pair of long strands between domains II and IV. The four domains and the pair of connecting strands enclose a 28 Å hole. The active site tyrosine has been identified to be Tyr-319 (4). It is located in domain III at the interface between this domain and domains I and IV. This strategic position suggests that during the catalysis of DNA breakage, passage, and rejoining, domain III on one side and domains I and IV on the other are likely to undergo large relative movements (15). Following the formation of the covalent adduct, for example, the lid is probably lifted away from the base to allow the passage of a second strand (15). The structural features of the 67-kDa fragment and the homology alignment of the amino acid sequences of the type IA enzymes provided a useful backdoor for the studies described below.

EXPERIMENTAL PROCEDURES
Site-directed Mutagenesis—Alanine substitution mutants were constructed by site-directed mutagenesis, using a commercial kit and following the protocol of the supplier (CLONTECH). The E. coli DNA topoisomerase I overexpression plasmid pJW312 (16) was used in these constructions. A selection primer, which changes a unique Scal site within the β-lactamase gene of the plasmid to a SauI site, and mutagenesis primers for the intended alanine codon substitutions, were purchased from commercial suppliers. The nucleotide sequences of the mutagenesis primers used in the construction of the E93A, H33A, D111A, E115A, T318A, Q309A, T322A, D323A, H365A, and T496A were, respectively, 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', and 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3'. The oligonucleotides used in the construction of E9Q and H365A, and T496A were, respectively, 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3', and 5'-CTT-GTC-ATA-GTG-GCT-A-GC-GGC-TAT-ATC-GAC-CGG-GCA-GAC-TCC-AC-3'.

RESULTS
Mutagenesis of Highly Conserved Amino Acid Residues in E. coli DNA Topoisomerase I—To identify amino acid side chains of E. coli DNA topoisomerase I that might be directly involved in the catalysis of DNA breakage and rejoining, 12 point mutants, E99A, E99S, D111A, E115A, Q309A, E313A, T318A, Q309A, T322A, D323A, H365A, and T496A, each designated by the particular amino acid residue replaced by alanine, were constructed by site-directed mutagenesis. An additional mutant T319A was also constructed for comparison with the others, because inactivation of the enzyme by this mutation was anticipated from the known function of Tyr-319 in catalysis (4), as well as from previous studies of the Y319F and Y319S mutant enzymes (23).

In selecting the residues for alanine substitution, it was assumed that a particular catalytic residue must be present at corresponding positions in all members of this subfamily and that it must possess a polar side chain, which is likely to be involved in the catalysis of DNA breakage and rejoining. Variability in residues at a conserved position was deemed acceptable only if all residues at the position contained a similar chemical group: interchanges among aspartate, glutamate, asparagine, and glutamine at a particular position in the homology alignment, for example, were considered acceptable because of the presence of a carboxyl group in each of the residues; on the other hand, a highly conserved amino acid residue such as Tyr-319 was considered to be an unlikely candidate because of the presence of a phenylalanine at this position in Sulfolobus acidocaldarius reverse gyrase (38). Several amino acid residues, including Lys-18, Arg-209, Tyr-91, and Ghu-547, which fulfill the criterion of being functionally conserved, were not selected for mutagenesis because of their relatively distant locations from the active site tyrosine Tyr-319 (15). The locations of Tyr-319 and the other 12 amino acid residues selected for alanine substitution mutagenesis are shown in Fig. 1B.
Relaxation of Negatively Supercoiled DNA by Wild-type and Mutant Enzymes—Wild-type *E. coli* DNA topoisomerase I and the 13 alanine substitution mutant proteins were individually overexpressed in ΔtopA cells harboring plasmids encoding the proteins. All mutant proteins were found to be expressed to a high level comparable with that of the wild-type enzyme. Each of the overexpressed proteins was purified to homogeneity and assayed for its ability to relax negatively supercoiled DNA. In Fig. 2, each set of four lanes represents assays in which the concentration of the topoisomerase was successively diluted 5-fold each time from left to right.

Although all mutants were constructed by substituting a highly conserved polar residue by alanine, the majority of these were found to be catalytically active. In addition to Y319A, in which the active site tyrosine was replaced by alanine, only E9A and R321A were found to have no detectable DNA relaxation activity.

The above findings led to the construction of two additional mutants: E9Q, in which Glu-9 is replaced by a glutamine, and R321K, in which Arg-321 is replaced by a lysine. The lysine substitution mutant R321K was found to be as active as the wild-type enzyme in the removal of DNA negative supercoils, but the E9Q mutant enzyme showed little activity in comparison with the wild-type enzyme (results not shown).

Formation of Covalent Adduct between Mutant *E. coli* DNA Topoisomerase I and DNA—Fig. 3 illustrates the results of a typical experiment in which covalent adduct formation between DNA and wild type (lane 1), E9A (lane 2), E9Q (lane 3), Y319A (lane 4), and R321A mutant enzyme (lane 5) was examined. A 388-base pair-long DNA fragment uniquely labeled at a 5’ end was used in this experiment. The fragment was heat denatured and incubated with wild-type or mutant *E. coli* DNA topoisomerase I in 40 mM Tris-HCl, pH 7.5, 10 mM KCl, and SDS was then added to 1%. As shown previously, protein-DNA covalent adduct formation is accompanied by cleavage of single-stranded DNA at sites that are determined by both structural and sequence features (24, 25). There is a strong preference of a cytosine at position −4, that is, four nucleotides...
upstream of the cleavage site (2). In the case of wild-type *E. coli* DNA topoisomerase I (*lane 1* of Fig. 3), the specificity of the cleavage reaction resulted in a distinctive distribution of cleavage products (compare the pattern of the *lane 1* sample with that of the untreated control run in *lane 6*). For the two mutant proteins E9A and H33A that showed little DNA relaxation activity (Fig. 2), the former showed no cleavage activity (*lane 2* of Fig. 3), whereas the latter showed reduced but significant level of DNA cleavage (*lane 5* of Fig. 3). In contrast to the E9A mutant, the ESQ mutant protein showed full cleavage activity (*lane 3* of Fig. 3). In some cleavage assays, the reaction mixtures also contained 0.1 mM EDTA; no difference in the cleavage patterns was observed by the inclusion of this metal chelating agent.

As expected, the active site tyrosine mutant Y319A showed little DNA cleavage activity (*lane 4* of Fig. 3). Careful inspection of the autoradiogram revealed, however, the presence of faint bands corresponding to cleavages at a subset of the cleavage sites of the wild-type enzyme. The significance of these bands will be discussed in a later section.

Experiments similar to the one shown in Fig. 3 were carried out for the other mutant enzymes. As expected, mutant proteins that showed DNA relaxation activity comparable with that of the wild-type enzyme were found to cleave DNA with efficiencies and site preferences similar to those of the wild-type enzyme (data not shown).

**DNA Rejoining by Mutant *E. coli* DNA Topoisomerase I**—For the complex between single-stranded or negatively supercoiled DNA and bacterial DNA topoisomerase I, it is known that addition of excess salt leads to the dissociation of the complex to give DNA with intact strands (21, 22). Prior to salt addition, a fraction of the enzyme-DNA complex is presumably in the form of the covalent intermediate that can be revealed by the addition of a protein denaturant. The addition of salt to the enzyme-DNA complex therefore appears to drive the dissociation of the enzyme and the rejoicing of the DNA. In the absence of added Mg(II) and in the presence of excess EDTA, however, a significant fraction of the enzyme, termed the “salt-stable complex,” was found to remain bound to single-stranded DNA upon addition of molar amounts of salt (21, 22).

The salt-induced reversal of DNA cleavage was exploited to test whether a mutant enzyme that showed DNA cleavage activity might be deficient in rejoicing the broken DNA; following the DNA cleavage reaction by a mutant enzyme blocked in its DNA rejoicing activity, the addition of salt would not be expected to rejoin the cleaved DNA. *Lanes 1–3* of Fig. 4 depict the results of such a salt-reversal experiment with wild-type *E. coli* DNA topoisomerase I. The *lane 1* sample in Fig. 4 was treated in the same way as that analyzed in *lane 1* of Fig. 3. A DNA fragment, 32P-labeled at a unique 5’ end was denatured and incubated with the *E. coli* enzyme. SDS was then added to a final concentration of 1% to denature the enzyme and to reveal the formation of the covalent complex. For the sample run in *lanes 2* and *3*, incubation of the denatured DNA and the *E. coli* enzyme was carried out in the usual manner. Before the addition of SDS, however, NaCl was added to the *lane 2* sample to a final concentration of 0.8 M, and NaCl and MgCl₂ were added to the *lane 3* sample, to 0.8 and 10 mM, respectively. The pattern shown in *lane 3* was expected from results of the earlier studies; exposure of the enzyme-DNA complex to high salt would lead to rejoicing of any cleaved DNA and dissociation of the enzyme from the DNA. Thus in contrast to the sample run in *lane 1*, which showed substantial amounts of cleaved DNA, the bulk of the DNA in the *lane 3* sample appeared uncleaved.

The high salt-induced rejoining of DNA was also observed in the absence of added Mg(II) (*lane 2*). When EDTA was added to 10 mM before the addition of NaCl, however, rejoicing was largely abolished unless Mg(II) was added to a concentration of

**Fig. 2. Relaxation of a negatively supercoiled DNA by wild-type *E. coli* DNA topoisomerase I and its various alanine substitution derivatives.** A set of four assay mixtures are shown for each enzyme specified in the *top margins*. Each assay mixture contained, in a total volume of 20 μl, 40 mM Tris-HCl, pH 7.5, 10 mM KCl, 2 mM MgCl₂, and 400 ng of negatively supercoiled pKS+ (Stratagene). The amounts of the enzyme were 100, 20, 4, and 0.8 ng, respectively, in each quartet of samples from left to right. After incubation at 37 °C for 20 min, Na₃EDTA was added to a final concentration of 100 mM to quench the reaction. The quenched reaction mixtures were analyzed by electrophoresis in a 0.7% agarose gel slab in 50 mM Tris-borate and 1 mM EDTA. Following electrophoresis, the gel was stained with 1 μg/ml ethidium bromide for 1 h, destained in water, and photographed over a UV light source.
1 mM or higher (data not shown) as observed previously (21, 6054).

Salt-induced reversal experiments with the mutant enzymes E9Q and R321A were shown in lanes 4–6 of Fig. 4, respectively. Similar to the case with the wild-type enzyme, the addition of salt to 0.8 M in either the presence or the absence of 10 mM Mg(II) was found to induce the rejoining of DNA by the catalytic pocket of the enzyme, and thus no significant rejoining with enzyme-linked DNA could occur following salt addition.

The DNA cleavage activities of the E9Q and R321A mutant proteins were also tested with a short DNA oligomer 5'-CAAT*GCGCT-3' known to be cleaved by E. coli DNA topoisomerase I at the position marked by an asterisk in the nonamer sequence (24). Similar to the results shown in Fig. 4 for the longer DNA substrate, the wild-type and the E9Q mutant protein were observed to cleave the DNA nonamer with comparable efficiency (lanes 2 and 5 of Fig. 5, respectively), and the R321A mutant protein showed a reduced level of cleavage activity (lane 8 of Fig. 5). In all cases the intensity of the labeled cleavage product remained constant, however, upon the addition of NaCl to 0.8 M (lanes 3, 6, and 9) to 0.8 and 10 mM, respectively. The samples were incubated for an additional 30 min before the addition of SDS to 1%. All samples were desalted by ethanol precipitation and then analyzed by electrophoresis in a 6% polyacrylamide DNA sequencing gel.

Both NaCl and MgCl₂ to 0.8 M and 10 mM, respectively (lanes 4, 7, and 10). These results were expected; the noncovalently bound cleavage product 5'-CAAT-3' would diffuse away from the catalytic pocket of the enzyme, and thus no significant rejoining with enzyme-linked DNA could occur following salt addition.

**Discussion**

We have applied alanine substitution mutagenesis in assessing the plausible roles of 12 residues, in addition to the nucleophile Tyr-319, in the catalysis of DNA breakage and rejoining by E. coli DNA topoisomerase I. Each of the residues was selected for site-directed mutagenesis based on the strict conservation of a polar group at that position in all homologues of phylogenetically diverse organisms including bacteria, eukaryae, and archaea. It is therefore surprising that only substitution at Glu-9 or Arg-321 was found to affect transesterification between the active site tyrosine Tyr-319 and the scissile DNA phosphorous. Alanine substitution at the other 10 highly conserved positions, including Asp-111 and Glu-115, showed little effect on the DNA relaxation activity of the enzyme. In the crystal structure of the 67-kDa fragment of E. coli DNA topoisomerase I (15), Asp-111, Asp-113, and Glu-115 form a triad that resembles the structure of a similar triad in DNA polymerase I, which binds one of the two Mg(II) ions that participate in exonucleolytic cleavage of DNA (26, 27). Asp-113 of E. coli DNA topoisomerase I was not selected for mutagen-
The mutagenesis approach itself has limitations in that it provides no information on the plausible involvement of backbone polar groups in the catalysis of DNA breakage and rejoining. Furthermore, it is also plausible that the strict sequence conservation criterion adopted by us in the selection of residues for mutagenesis might have overlooked essential side chains. Substitution of alanine for Arg-136, for example, was found to affect the DNA relaxation activity of the enzyme but not its DNA cleavage activity.\(^1\) Arg-136 was not selected in the mutagenesis work reported here because of uncertainties about its being strictly conserved. The ability of the mutant to cleave DNA suggests, however, that Arg-321 is not directly involved in the catalysis of DNA cleavage.

Despite the shortcomings, the results reported here provide several significant clues on the mechanism of type IA enzymes in general and \textit{E. coli} DNA topoisomerase I in particular. First, no amino acid side chain appears to fit the role of a general base in proton removal from Tyr-319. Glu-9, being in domain I of the 67-kDa protein crystal structure, appears to be too far from the active site tyrosine Tyr-319, which is located in domain III. This is especially so in view of the prediction that the two domains I and III would move away from each other upon the binding of DNA (15). Although mutating Arg-321 to alanine reduces the DNA relaxation activity of the \textit{E. coli} enzyme, the mutation has only a minor effect on DNA breakage (Figs. 3 and 4), and the mutant enzyme appears to be capable of rejoining the severed DNA strand (Fig. 4). The very high p\(K_a\) of an arginyl side chain also makes it an unlikely general base at neutral pH. Because Arg-321 can be replaced by lysine without a significant reduction in DNA relaxation activity (Fig. 2), it appears to participate as a monodentate group. This positively charged side chain probably interacts with an oxygen of the scissile phosphate, or it might have a minor effect on Tyr-319 deprotonation through stabilization of the negatively charged phenolate intermediate (28–30).

Second, because of the much higher p\(K_a\) value of a sugar hydroxyl relative to that of a tyrosyl hydroxyl, a well positioned proton acceptor is probably necessary to serve the role of a general base in proton removal from the 3' hydroxyl group during DNA rejoining and, similarly, that of a proton donor during DNA breakage. From the results reported here, Glu-9 appears to be the best candidate for these roles. Either by itself or through a bridging water, Glu-9 could serve a dual role of a proton donor and acceptor. Studies of the ES9 mutant protein suggest that such a dual role is apparently unaffected by the substitution of the glutamate at this position by glutamine.

The results shown in Fig. 4 raise questions on the role of Mg(II) in DNA cleavage and rejoining. It is known that Mg(II) is required in the removal of DNA negative supercoils by \textit{E. coli} DNA topoisomerase I (7, 31) but not in the cleavage of DNA by the enzyme (2, 21). For the DNA rejoining reaction, a previous study showed that the transfer of an enzyme-linked DNA strand to the 3'-hydroxyl end of another DNA molecule requires Mg(II) (32), a finding that appears to contradict the results shown in Fig. 4, which suggest, superficially, that Mg(II) is not obligatory for intramolecular DNA rejoining.

This apparent conflict could be interpreted in two very different ways. In one view, Mg(II) is not required in the catalysis of either DNA breakage or rejoining by the \textit{E. coli} enzyme. According to this view, in the supercoiling removal reaction or the intermolecular rejoining reaction catalyzed by the enzyme, Mg(II) is mainly required for conformational changes in the enzyme-DNA complexes rather than for DNA strand cleavage and rejoining. The finding from fluorescence measurements that the binding of Mg(II) changes the environment of the tryptophan residues (33) is consistent with this interpretation.
These conformation changes in the enzyme-DNA complexes are most likely of key importance in the E. coli DNA topoisomerase I-mediated movements of DNA strands but may be less crucial in DNA strand breakage and rejoicing. A case in point is the finding with the E9Q mutant protein; it exhibits both DNA breakage and rejoicing activity, yet it shows little DNA relaxation activity.

In the other view, there is an intrinsic asymmetry in Mg(II) requirement in DNA breakage and rejoicing by E. coli DNA topoisomerase I; the divalent ion may be dispensable in the former, but it is required in the latter. In this view, the results in Fig. 4 could be attributed to either the presence of Mg(II) tightly associated with the enzyme or its inadvertent introduction when NaCl was added to 0.8 M in the salt-induced reversal experiment. Direct analysis of Mg(II) by atomic absorption spectroscopy showed no significant quantity of enzyme-bound Mg(II) in the preparation of enzyme used in the experiments reported in this work (datum not shown), which provides strong support of the notion that no Mg(II) is required in the DNA cleavage reaction (2, 21). In the salt-induced DNA rejoicing reaction, the amount of Mg(II) from added NaCl is estimated to be less than 10 μM at a NaCl concentration of 0.8 M from analytical data provided by the supplier (Sigma ultra grade NaCl). This level of contamination is an order of magnitude lower than the free Mg(II) concentration required for a stoichiometric ratio of one bound Mg(II) per E. coli DNA topoisomerase I (33). Nevertheless, it is plausible that the enzyme-DNA complexes might have a much higher affinity for Mg(II) than the free enzyme. The finding that salt-induced rejoicing of DNA cleaved by E. coli DNA topoisomerase I was largely absent in the presence of 10–20 mM EDTA, unless Mg(II) was added to 1 mM or higher, is consistent with the second interpretation. Whereas the available experimental data cannot rule out either interpretation, it seems clear that the concentration of Mg(II) required for removal of negative supercoils is much higher than that for DNA rejoicing; thus one important role of Mg(II) is to facilitate conformational changes in the enzyme-DNA complexes.

Finally, it is noteworthy that in the study of DNA cleavage by the mutant Y319A, faint but discernible cleavage products were seen (Fig. 3, lane 4). These products could be attributed to the presence of a low amount of wild-type enzyme in the purified mutant protein, because the mutant protein used in the cleavage experiment was purified from strain BL21-topA cells overexpressing a plasmid-borne topA (Y319A). Such an explanation seems unlikely, however. For multiple preparations of the mutant proteins Y319A, E9A, and R321A from strain BL21 cells, only Y319A consistently showed low amounts of cleavage products (compare the patterns of lanes 2, 4, and 5 of Fig. 3). A more plausible interpretation is therefore that in the absence of a bulky tyrosyl group at position 319, a solvent water molecule could occupy this location and serve as a nucleophile in DNA cleavage.

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