Identification of Active Site Residues in the “GyrA” Half of Yeast DNA Topoisomerase II*

Qiyong Liu and James C. Wang‡

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

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‡To whom correspondence should be addressed: Dept. of Molecular and Cellular Biology, Harvard University, 7 Divinity Ave., Cambridge, MA 02138. Tel.: 617-495-1901; Fax: 617-495-0758.

Site-directed mutagenesis was carried out at 10 highly conserved polar residues within the C-terminal half of yeast DNA topoisomerase II, which corresponds to the A subunit of bacterial DNA gyrase, to identify amino acid side chains that augment the active site tyrosine Tyr-782 in the breakage and rejoining of DNA strands. Complementation tests show that alanine substitution at Arg-690, Asp-697, Lys-700, Arg-704, or Arg-781, but not at His-735, His-736, Glu-738, Gln-750, or Asn-828, inactivates the enzyme in vivo. Measurements of DNA relaxation and cleavage by purified mutant enzymes show that these activities are abolished in the R690A mutant and are much reduced in the mutants D697A, K700A, R704A, and R781A. When a Y782F polypeptide with a phenylalanine substituting for the active site tyrosine was expressed in cells that also express the R690A polypeptide, the resulting heterodimeric yeast DNA topoisomerase II was found to nick plasmid DNA. Thus in a dimeric wild-type enzyme, Tyr-782 in one protomer and Arg-690 in the other cooperate in trans in the catalysis of DNA cleavage. For the residues D697A, K700A, R704A, and R781A, their locations in the crystal structures of type II DNA topoisomerase fragments suggest that Arg-781 and Lys-700 might be involved in anchoring the 5’ and 3’ sides of the broken DNA, respectively, and the roles of Asp-697 and Arg-704 are probably less direct.

Because of the double helix structure of DNA, its cellular transactions often require the passage of individual DNA strands or double helices through one another. The DNA topoisomerases are enzymes that have evolved to fulfill such requirements. The type I enzymes catalyze DNA strand passage by transiently breaking one DNA strand at a time, and the type II enzymes create transient breaks in both strands of a DNA segment for the enzyme-mediated passage of a second DNA segment (for reviews, see Refs. 1–3 and references therein). The type I enzymes are further divided into two subfamilies IA and IB that are distinct in terms of their catalytic characteristics and amino acid sequences (3). Although the type II enzymes were thought to form a single subfamily, recent studies suggest that the enzyme DNA topoisomerase VI from archaeal hyperthermophiles may represent a separate subfamily (4).

DNA topoisomerases catalyze the breakage of DNA strands via transesterification between an enzyme tyrosyl group and a DNA backbone phosphoryl group, forming a phosphotyrosine link between the two and leaving a deoxyribosyl hydroxyl group on the other end of the broken DNA strand (5–11). Rejoining of the strand occurs via an apparent reversal of the DNA cleavage reaction; the hydroxyl group formed in the cleavage reaction acts as the nucleophile in a second transesterification, breaking the phosphotyrosine link and reforming the DNA phosphodiester bond. The rejoining reaction could be the exact microscopic reversal of the breakage reaction; conformational changes in the enzyme between the DNA cleavage and rejoining steps are plausible, however, and thus the two reactions could involve transition states that are not identical (12).

For the type II DNA topoisomerases, sequence alignments of polypeptides other than those of the archaeal DNA topoisomerase VI class show a high degree of homology (see for example, Ref. 13). All type II DNA topoisomerases act as homodimers of 1–3 subunits (14–19), and they create transient openings or gates in a double-stranded DNA through transesterification between a pair of active site tyrosyl residues in each enzyme and a pair of phosphates 4 base pairs apart in the DNA (18, 20, 21). These enzymes utilize ATP in their transport of a second DNA segment through such a transiently opened DNA gate (1–3). According to the two-gate protein clamp model (22, 23), a homodimeric type II enzyme bound to a DNA segment (the gate or G segment) acts as an ATP-modulated protein clamp. A second DNA segment (the transported or T segment) can enter an open entrance gate of the protein clamp. The binding of ATP to the enzyme triggers the closure of the entrance gate, and this closure in turn causes a cascade of reactions: the entered DNA segment is forced through a transient opening in the DNA G segment created by the enzyme and expelled through the exit gate of the enzyme (22, 23).

The crystal structure of a 92-kDa fragment of yeast DNA topoisomerase II spanning amino acid residues 409–1201 was reported in 1996 (24). The region of the single polypeptide yeast enzyme from amino acid residues 409 to about 660 corresponds to the C-terminal half of the B subunit or GyrB2 protein of Escherichia coli DNA gyrase and has been termed the B’ subfragment; the region of the yeast enzyme extending from the end of the B’ subfragment to around residue 1200 corresponds to the N-terminal two-thirds of the A subunit or GyrA protein of E. coli DNA gyrase and has been termed the A’ subfragment. The 92-kDa yeast B’A’ fragment forms a homodimer in the crystal and in solution, and this dimeric protein has been shown to cleave double-stranded DNA to form a pair of protein-DNA covalent links (24). The last result shows that all residues involved in DNA breakage and rejoicing are located within the yeast (B’A’)2 protein.

Examination of the 92-kDa structure led to the suggestion
Identification of Active Site Residues

Adjacent codon triplets are separated by a space for clarity, and differences in nucleotides from the original sequence are indicated by boldface letters. The restriction site added or removed by the introduction of a particular mutagenic oligonucleotide is specified in the right-hand column (plus for addition and minus for removal), and the restriction site added or removed is underlined in each sequence.

| Mutant | Oligonucleotides (5'-3') | Restriction site alteration |
|--------|--------------------------|-----------------------------|
| R690A  | G GCC GAT AAT ATA GCA TCG ATT CCC AAC T G +Cia1  |
| D302A  | CCC ATT GTA TTA GCC GAA TAA TAA GCT GGC +Msp1  |
| R700A  | GAT GGA TTT CCC GGG CAA AAG AAA GGT C +Smal |
| R704A  | GAT GGA TTT AAA CCC GGG CAA GAA AAA GGT C +Msp1  |
| H735A  | TGT AGC GCA TAT GGG CAT GTT GAG CAG +FspL–NcoI |
| H736A  | CG CAT TAT CAC GGT GAG GAC TC -NcoI |
| E738A  | T CAT CAG GGT GAA TCA TGG C +NotI |
| Q750A  | ATT ATT GGG CTT GCC GCA AAC TTT GTT GG +NotI |
| R781A  | GAT GCA GCT GCC GGC GAA TAT ATC TAC ACA G -NcoI |
| N828A  | CT ATG ATT CTT GTT GCC GGT GCT GAG -HpaI |

that the conformation of the polypeptide in this structure corresponds to that of the yeast enzyme after it has cleaved the DNA G segment and pulled the enzyme-linked DNA ends apart for the passage of the T segment through the widened opening (24). A pair of short DNA helices, each with a four-nucleotide single-stranded extension at a 5’ end, has been modeled into a DNA-binding site in each half of the (B‘A’)2 protein (24). This structural model is supported by recent protein footprinting experiments in which the effects of DNA binding on the citrullination of individual lysyl side chains in the yeast enzyme were measured (25).

Recently, the crystal structure of a 59-kDa fragment of E. coli GyrA protein, corresponding to the A’ portion of the 92-kDa yeast fragment, was reported (26). This structure is believed to resemble closely the conformation of the polypeptide in a DNA-bound enzyme before the DNA is cleaved by the enzyme (26). Based on the distribution of surface charges and the positions of the active site tyrosines in the crystal structure, a curved DNA segment has been modeled into the structure (26). Whereas there are significant differences in the spatial arrangements of the various domains within the A’ subfragment in the yeast (B‘A’)2 crystal structure and their counterparts in the 59-kDa E. coli GyrA fragment crystal structure, the remarkable conservation of the overall architecture of the polypeptide in the two structures provides strong support of the notion that these enzymes act similarly in their ATP-dependent transport of one duplex DNA segment through another.

The crystal structures of the yeast DNA topoisomerase II 92-kDa fragment and the E. coli DNA GyrA 59-kDa fragment have provided much needed structural information for understanding the mechanism of DNA breakage/rejoining by the type II DNA topoisomerases. To gain further mechanistic insight on the reactions they catalyze, we have applied site-directed mutagenesis in the search of residues that are involved in the catalysis of DNA cleavage and rejoining. In the present communication, we report mutagenesis analysis of the A’ subfragment of yeast DNA topoisomerase II.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—A 6.4-kb plasmid pSW201 bearing the yeast TOP2 gene encoding DNA topoisomerase II was used in the construction of the alanine substitution mutants, using a commercial kit and following the experimental protocol of the supplier (CLONTECH). The sequences of the mutagenic oligodeoxyribonucleotides used in the various constructions are listed in Table I. To facilitate the identification of the desired mutants, each mutagenic oligomer was designed to alter the restriction pattern of the parental plasmid in addition to introducing the desired codon change. The oligomer 5'–GAAAAGTGGCCACCTGAGCCTAAGAACACATTATTAC-3', which was previously designed to change a unique AatII site in pSW201 to the underlined SacI site (27), was used as the selection primer in the construction of the mutants. Mutant plasmids were screened by digestion with appropriate restriction enzymes that cut at the altered sites, and final confirmation of the mutations was done by direct nucleotide sequencing of the altered regions.

Two sets of expression plasmids were constructed from the mutagenized pSW201 derivatives. For genetic complementation tests of the mutants, the KpnI-AvrII fragment from each of the mutated derivative of pSW201 was used to replace the corresponding wild-type TOP2 fragment in YEpTOP2-PGAL1, a multicopy plasmid used previously in the overexpression of full-length yeast DNA topoisomerase II from an inducible yeast GAL1 gene promoter (9). For overexpression of the mutant enzymes for biochemical characterization, the KpnI-AvrII fragment from each of the mutated pSW201 was used to replace the corresponding fragment in pGALITop2-1–1196-HMK-(His)6, in which the first 1196 codons of the wild-type yeast TOP2 gene are fused to two short runs of codons, one encodes a phosphorylation site of heart muscle kinase and the other a stretch of six histidines (25). The tagged enzyme expressed by pGALITop2-1–1196-HMK-(His)6 has previously been shown to be fully active in vitro (25, 28), and the presence of the six histidines at the C terminus facilitates the purification of the enzyme on Ni-NTA resin (Novagen).

Complementation Tests—YEpTOP2-PGAL1 and its mutated derivatives were individually transformed into yeast strain JCW26 carrying the temperature-sensitive allele top2-4 (29) or strain CH34 carrying the cold-sensitive top2-13 allele (30). One single colony of a strain JCW26 transformant was picked and suspended in 1 ml of water. Following a serial dilution of the cell suspension, equal volume aliquots of the final dilutions were plated in duplicates. One set of plates was incubated at 25 °C and the other set at 35 °C for 72 h. Complementation test with strain CH34 top2-13 (30) was done similarly, but the two sets of plates were incubated at 25 and 13 °C.

Overexpression and Purification of the Wild-type and Mutant Proteins—Overexpression of the plasmid-borne Top2-1–1196-HMK-(His)6 and various alanine substitution derivatives was carried out in a protease-deficient yeast strain BCY123 (originally obtained from the laboratory of R. Kornberg, Stanford University) or JEL1 (31) according to the procedures previously described for overexpression of the full-length yeast enzyme (9). Single colonies of transformants were picked and inoculated in minimal medium lacking uracil and supplemented with 3% (v/v) glycerol, 2% (v/v) lactic acid, and 2% (w/v) glucose. Cultures were grown at 30 °C for 24 h to reach a cell density of 0.4–1.5 × 108. These diluted by 100-fold into YPD medium (32) in 2-liter flasks, and growth of cells was resumed by shaking the flasks on an orbital shaker platform in a 30 °C incubator. When the cell density of a culture climbed back to 0.4–1.5 × 108 (in about 24 h), galactose was added to a final concentration of 2% (w/v) to induce the expression of the plasmid-borne top2 gene from the GAL1 promoter. Cells were harvested by centrifugation 8–9 h post-induction. The pellets were washed immediately for the next step or were flash-frozen with liquid N2 and stored at −70 °C until use. Freshly pelleted cells or cells thawed from the frozen pellets were resuspended in an equal volume of 1 × Ni-resin binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, pepstatin A, and aprotinin) and vortexed vigorously with glass beads in 50-ml conical plastic tubes (9, 33). Cell lysate was clarified by centrifugation and loaded on a Ni-resin column (Novagen, His-Bind Resin), following the manufacturer’s protocol with minor modifications. The column was thoroughly washed with 1× binding buffer and then 1× binding buffer + 25 mM imidazole, and the hexahistidine-tagged protein was eluted by increasing the imidazole concentration to 1 M. The salt concentration in the protein eluate was reduced to 100 mM by the
addition of 5 mM Tris-HCl, pH 7.7, 10 mM 2-mercaptoethanol, 10% glycerol (v/v), and 1 μg/ml each of leupeptin, pepstatin A, and apronitin. The diluted protein was then concentrated by loading on a small heparin column (about 200 μl for 1 mg of protein) and eluting with the dilution buffer plus 45 mM Tris-HCl, pH 7.7, and 650 mM KCl. Drops were collected, and the peak fractions were pooled and mixed with equal volume of 80% (v/v) glycerol for storage at −70 °C. During the concentration step, some contaminant proteins were also removed.

Preparation of the R690A-Y782F Heterodimeric Protein—The yeast strain JEL1(Stop1), a derivative of JEL1 constructed by targeted disruption of the TOP1 gene encoding DNA topoisomerase I, was transformed with both the R690A derivative of pTop2-(1–1196)-HMK-(His)₆ and a second plasmid pJEL131-Y782F, which expresses full-length yeast DNA topoisomerase II with a Y782F mutation. The Y782F mutation in pJEL131-Y782F was constructed by site-directed mutagenesis of pJEL131, which was derived from YEpTOP2-PGAL1 by replacing the URA3 marker in it with a LEU2 marker (34). Induction of the GAL1 promoter-linked genes and the preparation of cell extracts were carried out as described above. Because of weaker binding of the heterodimeric protein to the Ni-resin, presumably owing to the presence of only one hexahistidine tag in a heterodimeric molecule, the procedure used in the purification of the other hexahistidine-tagged derivatives of yeast DNA topoisomerase II was slightly modified. After loading the protein on the Ni-resin, the column was thoroughly washed with only the 1× binding buffer before eluting the protein with the same buffer plus 1 M imidazole.

RESULTS

Selection of Amino Acid Residues for Site-directed Mutagenesis.—There are a large number of highly conserved amino acid residues in the type II DNA topoisomerases from a diverse collection of organisms ranging from T-even phage to human.

In order to focus on residues that are likely to participate in the catalysis of DNA breakage and rejoining, we applied the same criteria used in a recent study of E. coli DNA topoisomerase I (35). It is assumed that these residues are likely to possess polar side chains and that variations at a conserved position in the above considerations led to the construction of 10 mutants within the C-terminal or “GyrA” half of yeast DNA topoisomerase II (25, 28). To avoid cumbersome notations, in the biochemical experiments reported below the C-terminally tagged protein with amino acid residues 1–1196 of the wild-type yeast DNA topoisomerase II is simply referred to as the “wild-type” enzyme, and the same fusion protein with a mutation within residues 1–1196 of the yeast enzyme is referred to as a mutant enzyme.

Fig. 2 depicts the results of a set of experiments designed and carried out based on preliminary assays of the various enzyme preparations. In each assay, a negatively supercoiled 3-kb plasmid was incubated with the wild-type enzyme or one of the mutant enzymes under standard assay conditions, and the reaction mixture was deproteinized for analysis by agarose gel electrophoresis. In the top margin above each lane of the gel slab, the molar ratio of enzyme to DNA (E/DNA) in the assay mixture analyzed is specified, and the presence or absence of ATP in the assay mixture is indicated by a plus or minus sign. In the incubation mixture without enzyme (leftmost lane), the majority of the DNA migrated at the position of the supercoiled form (bottom band), and a smaller amount of the DNA migrated as nicked DNA rings (upper band). For the set of four samples incubated with the wild-type enzyme (lanes below WT in Fig. 2), in the absence of ATP the electrophoretic pattern of the DNA remained unchanged from that of the no-enzyme control at a high E/DNA molar ratio of 32 (see the leftmost lane of the set), indicating the absence of any ATP-independent DNA relaxation activity or endonuclease activity in the enzyme preparation used. In the presence of ATP, the wild-type enzyme relaxed the DNA effectively; complete relaxation was evident at an E/DNA ratio as low as 1/32, and partial relaxation of the DNA was seen at an E/DNA ratio of 1/128 (see the two middle lanes of the set). In contrast, the mutant enzyme R690A showed no detectable activity at an E/DNA ratio as high as 128.
Thus the DNA relaxation activity of the R690A mutant enzyme is at least a factor of $128 \times 128$ or 4 orders of magnitude lower than that of the wild-type enzyme.

The four mutant enzymes K700A, R704A, R781A, and N828A showed reduced but readily detectable DNA relaxation activity. The drop from the relaxation activity of the wild-type enzyme, estimated to be about 1–2 orders of magnitude for the four, is substantial. Assays of the DNA relaxation activity of the D697A mutant enzyme were not included in Fig. 2. The yield of this protein was lower than those of the others by more than an order of magnitude, and this poor yield might reflect improper folding of the mutant protein. The ATP-dependent DNA relaxation activity of purified D697A protein was detectable only at rather high $E/DNA$ ratios and was estimated to be 3 orders of magnitude lower than that of the wild-type enzyme (results not shown).

**Covalent-Adduct Formation between Mutant DNA Topoisomerase II and DNA**—To test the possibility that a mutant enzyme without DNA relaxation activity can nevertheless cleave DNA via transesterification, or the possibility that a mutant enzyme with a reduced level of DNA relaxation activity may possess a normal level of DNA cleavage activity, the purified wild-type and mutant enzymes were assayed for their cleavage of plasmid DNA.

In these experiments, the enzyme to DNA molar ratio was fixed at 3:1. In each set of four samples analyzed in the agarose gel slab shown in Fig. 3, 0.5 mM etoposide, a topoisomerase II-targeting anticancer drug known to stabilize the covalent enzyme-DNA adduct (37), was present in samples 1 and 2, and 1 mM ATP was present in samples 1 and 3. For samples treated with the wild-type enzyme (below WT), extensive cleavage of the input DNA was seen in the presence of both etoposide and ATP (lane 1 of the WT set); most of the supercoiled form of the input DNA was converted to the linear form at the position marked by $L$ in the right margin of the figure, and the diffuse streak of DNA below the linear DNA band signifies that a
fraction of the input plasmid DNA had been cut more than once. In the presence of etoposide but without ATP (lane 2 of the WT set), DNA cleavage was less extensive, in agreement with the known effect of ATP in the trapping of the covalent enzyme-DNA intermediate by etoposide (37). In the absence of etoposide, only a trace of linear DNA was formed at the E/DNA ratio employed in the presence or absence of ATP (lanes 3 and 4 of the WT set). The covalently closed DNA species in the samples analyzed in lanes 3 and 4 migrated to different positions (indicated by R and S, respectively, in the right margin of Fig. 3), owing to the relaxation of the input DNA by the wild-type enzyme in the presence of ATP.

The R690A mutant enzyme, which is unable to relax supercoiled DNA (Fig. 2), exhibited no DNA cleavage activity (Fig. 3, lanes below R690A). The R697A mutant enzyme similarly showed no detectable linear DNA product at an E/DNA ratio of 3:1 (result not shown). Mutant enzymes K700A, R704A, R781A, and N828A, which showed partial DNA relaxation activity, were found to cleave DNA in the presence of etoposide and ATP (lane 1 in each of the mutant enzyme panels in Fig. 3) but at a level significantly below that of the wild-type enzyme. As observed with the wild-type enzyme, during the DNA cleavage assays relaxation of the input supercoiled DNA by the K700A, R704A, R781A, or N828A mutant enzyme was evident in the presence of ATP and in the absence of etoposide (compare the electrophoretic mobilities of the covalently closed DNA in the lane 3 and lane 4 samples in each panel).

The R690A Enzyme Can Bind DNA and Form a Closed Enzyme Clamp upon Binding of a Nonhydrolyzable ATP Analog—To probe further why the R690A enzyme is incapable of either DNA relaxation or DNA cleavage, experiments were carried out to test its ability to bind DNA and undergo the ATP-modulated closing of the protein clamp. As shown previously, binding of the nonhydrolyzable ATP analog ADP-PPNP to yeast DNA topoisomerase II converts the enzyme to a closed-clamp conformation through dimerization of the N-terminal ATPase domains of the dimeric enzyme (22, 23). For an enzyme molecule bound to a DNA ring, this conversion introduces a topological link between the annular enzyme and the DNA ring, forming a complex characterized by its salt stability (22, 23).

Fig. 4 depicts the results of a representative series of experiments. In the control experiment under the heading None, a 3-kb plasmid was incubated with ADP-PPNP in the absence of
Identification of Active Site Residues

DNA topoisomerase II, and NaCl was then added to a final concentration of 1 mM before filtering the mixture through a glass-fiber filter, which would retain protein-bound DNA (36). The filter was washed with the same buffer containing 1 mM NaCl. The high salt wash and filtrate were combined, and ethanol was added to the mixture to precipitate the DNA for gel electrophoresis (lane 1). The filter was then eluted with 0.5% SDS, and DNA recovered from the SDS eluate by ethanol precipitation was analyzed in lane 2. The same experiments were also carried out without the addition of ADPPNP, and DNA samples recovered from the filtrate and the SDS eluate in these were analyzed in lanes 3 and 4, respectively. As expected, either in the presence or absence of ADPPNP, the input DNA in the absence of DNA topoisomerase was mostly found in the filtrate/wash (lanes 1 and 3) and was undetectable in the SDS eluate (lanes 2 and 4).

The samples analyzed in lanes 1–4 below WT were obtained in the same way as the corresponding samples described above for the control experiment, except that wild-type yeast DNA topoisomerase II was present in the incubation mixtures at an enzyme to DNA molar ratio of approximately 1. At this low E/DNA ratio, in the presence of ADPPNP the majority of the DNA was found in the filtrate/wash (lane 1). A significant amount of the input DNA was found, however, in the SDS wash (lane 2). This fraction represents DNA topologically locked in the closed-clamp conformation of the enzyme, as the protein and DNA remained associated in 1 mM salt, and the DNA was eluted from the filter only after treatment with a protein denaturant. In the absence of ADPPNP, the formation of a salt-resistant closed enzyme clamp does not occur, and no input DNA was recovered from the SDS eluate (lane 4). When the experiment with the wild-type enzyme was repeated at a higher E/DNA ratio (lanes 5–8 below WT), similar results were obtained. In the presence of ADPPNP, increasing the E/DNA molar ratio in the incubation mixture from 1 to 4 reduced the amount of input DNA in the filtrate/wash and increased the amount of the DNA in the SDS eluate (compare the patterns shown in lanes 1 and 5 and in lanes 2 and 6).

When the filter binding experiments in the presence and absence of ADPPNP were carried out with the R690A mutant enzyme (lanes 1–8 in Fig. 4 below R690A), the electrophoretic patterns of the samples were essentially identical to the corresponding ones with the wild-type enzyme. These results show that although the R690A mutant enzyme is incapable of removing DNA supercoils or undergoing transsterification with DNA, it can bind DNA and the DNA-bound enzyme can readily assume the closed-clamp conformation upon ADPPNP binding.

Cleavage of DNA—Together, the results shown in Figs. 2–4 suggest that the residue Arg-690 of yeast DNA topoisomerase II has a key role in DNA cleavage. In the R690A mutant enzyme, the closure of the N-gate upon ATP-binding appears unhindered, but DNA cleavage and relaxation apparently do not occur. This interpretation is supported by experiments on DNA cleavage by a heterodimeric enzyme with a Y782F mutation in one polypeptide and a R690A mutation in the other polypeptide. As will be described below, these experiments show that Arg-690 and Tyr-782 from different halves of a dimeric enzyme can cooperate in trans to cleave a DNA strand.

To prepare the heterodimeric enzyme, yeast cells were transformed with a pair of plasmids. The first plasmid was the same as that used for the overexpression of the R690A homodimeric protein with a C-terminal hexahistidine tag. The second was one that overexpresses full-length yeast DNA topoisomerase II with the active site tyrosine replaced by a phenylalanine. Following the induction of cells harboring the pair of plasmids, both heterodimeric and homodimeric enzymes were expected to form. The cellular level of the R690A polypeptide was found to be much higher than that of the Y782F polypeptide, as indicated by the relative amounts of the polypeptides in cell lysates (these polypeptides were readily resolved by SDS-polyacrylamide gel electrophoresis and quantified owing to their size difference). Purification of the hexahistidine-tagged proteins over Ni-resin yielded a mixture containing about 90% R690A homodimer and 10% R690A-Y782F heterodimer.

In Fig. 5, results of DNA cleavage by wild-type yeast DNA topoisomerase II and by the R690A-Y782F heterodimer and R690A homodimer mixtures were depicted. In samples analyzed in lanes 1–4 below WT, wild-type enzyme and DNA were mixed at a molar ratio of about 1 and were treated in exactly the same way as the WT samples 1–4 shown in Fig. 3. As expected, results identical to those described earlier were obtained. However, when the same experiment was repeated with
the R690A-Y782F heterodimer and R690A homodimer mixture, at a molar ratio of about 1 heterodimer per plasmid DNA (lanes 1–4 below Heterodimer in Fig. 5), nicking rather than linearization of the input DNA was observed in the presence of etoposide and ATP (lane 1). This nicking is expected to result from covalent adduct formation between DNA and a mutant enzyme molecule; the presence of covalently linked protein moiety in the nicked DNA is supported by a comparison of the electrophoretic mobilities of the nicked DNA products treated with SDS alone or with SDS and proteinase K (27) (results not shown). Because the R690A homodimer has no DNA cleavage activity (Fig. 3), it is clear that the unmodified residues Arg-690 and Tyr-782 in two separate polypeptides in the heterodimeric enzyme can cooperate to nick one of the two DNA strands in an etoposide- and ADPPNP-dependent fashion.

**DISCUSSION**

The solution of the three-dimensional structures of the 92-kDa yeast DNA topoisomerase II and the 59-kDa *E. coli* DNA GyrA fragment and the modeling of bound DNA into them have provided the much needed backdrop for deducing the roles of individual amino acid residues in the catalysis of DNA break-
age and rejoining by the type II DNA topoisomerases. It is particularly significant that the conformation of the protein in one structure is believed to represent that of a type II DNA topoisomerase after it has cleaved a DNA and pulled it apart (24), whereas that in the other the state of the enzyme prior to its cleavage of DNA (26).

The results reported in this work illustrate the importance of independent tests of conjectures based on structures and homology alignment of related enzymes. The polar residues His-735, His-736, Glu-738, and Gln-750 of yeast DNA topoisomerase II, for example, are highly conserved in a large number of type II DNA topoisomerases from bacteria, eukarya, and archaea, and their positions in the crystal structure of the 92-kDa yeast enzyme fragment are also suggestive of their involvement in DNA breakage and rejoining. Nevertheless, these residues can be replaced by alanine without affecting the functionality of the enzyme in vivo, indicating that they are nonessential in catalysis.

From the results reported here, within the GyrA region of yeast DNA topoisomerase II, alanine substitution at five positions, Arg-690, Asp-697, Lys-700, Arg-704, and Arg-781 (side chains colored red in Fig. 1, A and B), leads to dysfunction of the enzyme in vivo. Examination of Fig. 1, A and B, shows that Arg-781 and its neighboring active site tyrosine Tyr-782 (colored magenta) are located near the 5’-end of the DNA modeled into the protein structure, and Arg-690 and Lys-700 are posed near the 3’-end of the broken DNA. Before the enzyme-linked DNA ends are moved apart following DNA cleavage, however, the amino acid side chains near a pair of 5’ and 3’ ends derived from the same DNA strand must be close together. Therefore Arg-781 and Tyr-782 in one protomer are likely to be close to Arg-690 and Lys-700 of the other protomer of the same dimeric enzyme prior to the separation of the ends. Such a juxtaposed arrangement has also been implicated from the recently reported crystal structure of the 59-kDa E. coli GyrA fragment (26). In Fig. 6, A and B, the E. coli GyrA crystal structure with a DNA segment modeled into it is depicted (26). For ease of comparison, the positions of the gyrase amino acid residues corresponding to those of the yeast enzyme selected for mutagenesis in this work are shown by the same red and blue color coding, and their yeast counterparts are specified in parentheses. It is clear that in the 59-kDa GyrA fragment structure, Arg-121 (Arg-781) and Tyr-122 (Tyr-782) of one protomer and Arg-32 (Arg-690) and Lys-42 (Lys-700) of the other protomer form a patch near a DNA strand modeled into the protein. This patch of highly conserved amino acid residues has been suggested to represent the DNA cleavage and rejoining site of the enzyme (26). Therefore both crystal structures hint that in the DNA cleavage/rejoining reaction the active site tyrosyl group and the arginine side chain next to it are interacting with the 5’ side of the scissile phosphate, whereas a positively charged pair of residues, Arg-690 and Lys-700 in the yeast enzyme and Arg-32 and Lys-42 in E. coli GyrA protein, are interacting with the 3’ side of the scissile phosphate.

The present work underscores the key importance of Arg-690 of yeast DNA topoisomerase II (Arg-32 of E. coli DNA gyrase A subunit) in the breakage and rejoining of a DNA strand. The experiment shown in Fig. 5 provides strong evidence that the Tyr-782/Arg-690 pair in the yeast enzyme cooperates in trans in the cleavage, and presumably rejoining, of the DNA strand. Such a trans mechanism in DNA phosphoryl transfer has been previously shown for the yeast FLP recombinase (38–40), a member of a family of enzymes involved in site-specific recombination. In the DNA topoisomerase family, the type I enzymes act as monomers, and the type II enzymes act as dimers (3). The available structural and biochemical evidence suggests, however, that all DNA topoisomerases may share an important common feature for either a type I or a type II enzyme, catalysis of DNA breakage and rejoining are most likely to occur in an interdomainal cleft, with catalytically essential residues from at least two distinct domains. For the type IA enzyme E. coli DNA topoisomerase I, the crystal structure of a 67-kDa fragment has led to the suggestion that the active site tyrosine Tyr-319 in domain III of the fragment may cooperate with residues in domains I and IV in DNA cleavage and rejoining (41). In support of this conjecture, mutagenesis and biochemical studies indicate that Glu-9 in domain I has a key role in DNA strand cleavage and rejoining (35). For the type IB DNA topoisomerases yeast DNA topoisomerase I and vaccinia DNA topoisomerase, such interdomainal active sites has also been implicated (42, 43). A priori, placing active site residues in different domains should make it easier to move the ends of the DNA strand after the cleavage reaction and to ensure the correct positioning of these ends for their subsequent rejoining. The recently solved crystal structure of a fragment of phage λ integrase has added an additional example of bipartite active sites; the active site tyrosine Tyr-342 of the enzyme is located on a flexible loop about 20 Å from other essential residues (Ref. 44; see also Ref. 45 for a discussion on cis and trans mechanisms of DNA recombinases).

The locations of the pair of residues Arg-690 and Tyr-782 in the model of yeast DNA topoisomerase II depicted in Fig. 1 are suggestive that this key arginine side chain may interact with the oxygen bridging the scissile phosphorus and the sugar moiety in the DNA backbone, and Lys-700 may augment the positioning of the 3’ side of the scissile strand. The guanidino group of Arg-690 could stabilize the negatively charged 3’-oxygen during DNA strand breakage/rejoining through both hydrogen bonding and charge-pair formation. The nucleotide sequence of a Caenorhabditis elegans gene encoding a type II DNA topoisomerase indicates, however, that in the C. elegans enzyme the position corresponding to Arg-690 of the yeast enzyme is a glutamine (46). Thus either hydrogen bonding rather than charge-pair formation is the important factor, or a neighboring arginine in the structure of the C. elegans enzyme is fulfilling the role of Arg-690 in the yeast enzyme.

The roles of Asp-697 and Arg-704 of the yeast enzyme in the catalysis of DNA breakage and rejoining are less certain. The counterpart of Arg-704 in E. coli DNA gyrase, Arg-46 of the GyrA protein, has been suggested to serve a role of anchoring the 3’ end of the cleaved DNA together with Arg-47, which corresponds to Arg-705 of the yeast enzyme (26). Arg-705 of the yeast enzyme was not selected for mutagenesis in the present study because the selection criterion of conservation of a common chemical group is violated at this position by the presence of a phenylalanyl residue in phage T4 DNA topoisomerase (13). In the crystal structures depicted in Figs. 1 and 6, Arg-704 (Arg-46) and Asp-697 (Asp-39) form an ion pair, and the position of the highly conserved arginine appears to be too far from the DNA in the structural models for direct interaction with the scissile strand. Thus it appears that the primary role of the Arg-704 and Asp-697 pair is probably an indirect architectural one. The residue Asn-828 may serve a similar function.

The requirement of residues from both halves of a dimeric type II DNA topoisomerase in each of the DNA cleavage/rejoining sites also provides new insight in the design of inhibitors of this class of enzymes (for reviews, see Refs. 47 and 48 and references therein). Because the proper juxtaposition of these residues involves relative movements between the two enzyme halves, the binding of inhibitors to many different sites of the enzyme may interfere with the proper alignment of these res-
ides and therefore inhibit the catalytic function of the type II DNA topoisomerases.

Finally, it should be pointed out that although the A subunit of bacterial DNA gyrase has often been termed the “breakage-reunion subunit” of the enzyme because of the presence of the active site tyrosyl residue in it, neither Gyra by itself nor its yeast DNA topoisomerase II counterpart exhibits DNA cleavage/rejoining activity. On the other hand, DNA cleavage/rejoining activity is readily detected in E. coli in combination with the C-terminal half of Gyrb (49, 50), as well as in the yeast (B′A′)2 protein (24). Thus one or more residues within the B′ subfragment of yeast DNA topoisomerase II or its homologues must also be present in the catalysis of the breakage and rejoining of DNA strands. Mutagenesis studies of the B′ subfragment of yeast DNA topoisomerase II are being completed and will be reported at a later date.

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