The Role of Lysine 532 in the Catalytic Mechanism of Human Topoisomerase I*

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DNA topoisomerases are ubiquitous enzymes that solve topological problems generated by key nuclear processes such as DNA replication, transcription, recombination, DNA repair, chromatin assembly, and chromosome segregation by catalyzing the passage of individual DNA strands or double helices through one another. All cells contain two highly conserved classes of topoisomerases that are differentiated on the basis of their mechanistic and physical properties. The monomeric type I enzymes do not require high energy cofactors to change the topology by passing an intact helix through a transient double-strand break they create in the same or different DNA (3). All type II topoisomerases and the type IA subfamily form transient covalent phosphodiester bonds with the 5’-end of broken DNA strands, whereas the type IB topoisomerases form a transient linkage to the 3’-end of the DNA (2).

The type IB topoisomerase subfamily includes eukaryotic topoisomerase I, the topoisomerases encoded by vaccinia and other poxviruses, and some newly identified bacterial enzymes (1, 4). Recently, a structure of human topoisomerase I with a bound 22-bp DNA has been solved by x-ray crystallography (5, 6). Human topoisomerase I is a monomeric protein of 765 amino acids that binds to double-stranded DNA in a "clamp"-like manner with a preference for supercoiled DNA (7). Both negative and positive supercoils can be relaxed without a requirement for a high energy cofactor such as ATP. The reaction involves nucleophilic attack of Tyr723 on a phosphodiester bond in the DNA, resulting in temporary breakage of one DNA strand and covalent attachment of the active site tyrosine to the 3’-phosphate at the nick. Based on information gained from the structure of the topoisomerase I-DNA complex, relaxation likely takes place by a rotational mechanism (6). Religation is mechanistically similar to strand breakage, with the 5’-hydroxyl at the nick acting as the nucleophile to reigate the DNA strand and release the enzyme.

The covalent intermediate between topoisomerase I and DNA is normally quite transient, i.e. strand relaxation is fast relative to strand cleavage. However, in the presence of the topoisomerase I-specific poison camptothecin (CPT),1 the rejoining step is slowed significantly, resulting in topoisomerase I being trapped in a covalent complex with DNA (8). The stalled topoisomerase I creates major problems for processes such as DNA replication, transcription, and recombination. In fact, the double-strand breaks resulting from a collision of a replication fork with the drug-trapped topoisomerase I are the predominant cytotoxic mechanism of this anti-cancer drug as evidenced by the protective effect of DNA synthesis inhibitors (9–11).

Addition of the protein denaturant SDS to a topoisomerase I reaction in vitro traps the covalent enzyme-DNA intermediate. This technique has proven useful for the determination of topoisomerase I cleavage sites on linear double-stranded DNA. The following weak consensus sequence was observed for the bases at positions −4 to −1 where cleavage occurs between the −1 and +1 positions, and the enzyme becomes attached covalently to the −1 nucleotide: (−4) A/T, (−3) G/C/A, (−2) T/A, and (−1) T (12, 13). The basis for this weak sequence prefer-

1 The abbreviations used are: CPT, camptothecin; topo70, NH2-termini truncation of human topoisomerase I missing first 174 amino acids; GST, glutathione S-transferase; DTT, dithiothreitol; WT, wild type; MES, 2-(N-morpholino)ethanesulfonic acid.
ence of topoisomerase I has not been determined, but a close examination of the crystal structure of human topoisomerase I bound to DNA reveals a possible mechanism (5, 6). Among the numerous interactions between the enzyme and its DNA substrate, only one residue makes a base-specific contact with the DNA. This contact is between the side chain amino group of Lys832 and the O-2 atom of the −1 thymine base. An analogous interaction between Lys832 and the −1 position on the DNA was observed in a crystal structure of human topoisomerase I with a DNA substrate that contains a cytosine at the −1 position, the next most preferred nucleotide at this position (12, 14). The fact that both pyrimidine bases interact with Lys832 through the O-2 raises the question of whether Lys832 might be important for the nucleotide preference of the enzyme at the −1 position.

The type IB topoisomerases share a common chemistry and three-dimensional structure with the tyrosine recombinases that include phage P1 Cre and Escherichia coli XerD recombinases, as well as the Int family of phage integrases (5, 15). The hallmark of the type IB topoisomerases and the tyrosine recombinases is an active site that consists of two arginines, one histidine, and the nucleophilic tyrosine (1, 16–18). A fifth essential catalytic residue, a lysine, has been identified biochemically in the XerD protein from Salmonella typhimurium (19), in Saccharomyces cerevisiae Flp (20), and in vaccinia topoisomerase (Lys167) (21). The equivalent residue in human topoisomerase I is Lys832. This lysine residue is conserved among type IB topoisomerases and tyrosine recombinases with an occasional conservative substitution of arginine at the corresponding position. Evidence has been presented that Lys832 in vaccinia topoisomerase acts as a general acid to protonate the leaving 5′-oxygen during the cleavage reaction (22). More recently Arg130 in the vaccinia enzyme has also been implicated in expulsion of the leaving group (23).

To further the role of Lys832 in catalysis by human topoisomerase I and in the sequence preference of the enzyme, we replaced Lys832 with alanine and, more conservatively, with arginine and analyzed the effects of these changes in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Cloning and Site-directed Mutagenesis—Mutant alleles of the NH2-terminally truncated human topoisomerase I (topo70) were generated using the QuickChange site-directed mutagenesis kit from Stratagene. The PCR products were performed on pGST-topo70 (24), using the following primer pairs to generate pGST-topo70K532R: K532Rforward (5'-GACTTCTCGGAAGACCTCATCAG) and K532Rreverse (5'-GCTGGAATGTTCGCGACGGCTGAC) and pFastBac1-topo70 K532A by replacing H632Q to WT. The resulting plasmids pFastBac1-topo70 K532R were then used according to the Bac-to-Bac expression system manual. The expression of the topo70 proteins was verified by immunoblotting. The procedures for baculovirus amplification, expression of topoisomerase I, and purification of the overexpressed proteins were described as previously (26).

Plasmid Relaxation Assay—Supercoiled plasmid pBluescript KSII (+) (Strategene) was used in the plasmid relaxation assays. These assays were performed essentially as described previously (26). All plasmid relaxation experiments were performed in a reaction buffer containing 150 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.1 mM DTT, and 0.1 mg of bovine serum albumin per ml. For the dilution experiment (Fig. 2A), proteins were serially diluted 2-fold in reaction buffer, and the reactions were initiated by the addition of 5 μl of the indicated amounts of the enzyme to a 15-μl sample containing 0.5 μg of supercoiled plasmid DNA. Reactions were incubated at 37 °C for 10 min and stopped by the addition of 5 μl of 5 × loading dye (2.5% SDS, 10% Ficoll, 50 mM EDTA, 0.125% bromphenol blue). The samples were then treated for 30 min at 37 °C with proteinase K (40 μg/ml) to remove topoisomerase I protein from any covalent complexes that were present. After electrophoresis in a 0.8% agarose gel in TBE (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA, pH 8), the gel was stained with ethidium bromide (0.5 μg/ml TBE), and bands were visible in Southern blots of nicked plasmid DNAs, and the DNA visualized with UV illumination. The time course assays were performed under the same conditions with the amounts of protein indicated in the figure legends.

Equilibrium Cleavage Assay—The 25-mer oligonucleotide CL25 (Fig. 3A) was 5′-end-labeled with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-32P]ATP and annealed to the complementary 25-mer CP25. Reaction mixtures containing 150 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 10 ng oligonucleotide substrate CL25/CP25 were incubated at room temperature (23 °C), and reactions were started by the addition of enzyme (200 nM). At the indicated time points, 20-μl samples were removed and the reactions stopped with 2% SDS. Half of these mixtures were added to 10 μl of protein sample loading buffer (156 mM Tris-HCl, pH 6.8, 5% SDS, 25% glycerol, 12.5% β-mercaptoethanol, 0.0025% bromphenol blue), boiled for 5 min, and 7 μl of each sample were analyzed by 10% SDS-PAGE. To the second half of the reaction, 2 μl of 1 M MgCl2 and 86 μl of ethanol were added to precipitate the DNA and the proteins that are covalently bound to it. The pellets were resuspended in 1 μl of 1.5 mg/ml trypsin and incubated at 37 °C for 1 h. Samples were then mixed with 15 μl of formamide loading dye (96% formamide, 20 mM EDTA, 0.03% xylene cyanol, 0.03% bromphenol blue) and boiled for 3 min, and 6-μl aliquots were analyzed by denaturing 20% polyacrylamide, 7% urea gel electrophoresis (sequencing gel). Image retrieval and analysis were carried out using a PhosphorImager in conjunction with the Imagequant software (Amersham BioSciences).

Suicide Cleavage Assays—The suicide cleavage assays were performed under conditions identical to those used for the equilibrium cleavage assays using as substrates CL14/CP25 (Fig. 4A) or CL22-SCP2 (Fig. 5A). The 14-mer CL14 or the 22-mer CL22-S were 5′-end-labeled and annealed to the complementary 25-mer CP25 or the 22-mer CP22, respectively, both of which had been cold-phosphorylated at their 5′-ends. Reactions were stopped with SDS and analyzed by both SDS-PAGE and sequencing gel electrophoresis as described above. The Imagequant software (Amersham BioSciences) was used for quantitation.

Religation Assay—The same labeled CL25/CP25 substrate and reagents used in the equilibrium cleavage assays were also used in the re-ligation assay. WT and K532R were incubated with the substrate for 10 min to ensure that equilibrium had been reached. K532A was incubated for 2 h to accumulate enough covalent complex that could easily be visualized by SDS-PAGE. At the 0 time point, NaCl was added to the reactions to a final concentration of 500 mM to prevent rebinding of DNA to topoisomerase I to the DNA after religation. Religation was assayed by the addition of SDS, and the samples were prepared for SDS-PAGE and sequencing gels as described for the cleavage assays.

Yeast Experiment—The yeast shuttle vector pHT-100 (27), kindly provided by O. Westergaard, allowed galactose-dependent topoisomerase I expression. It is a CEN/ARS plasmid that contains the 1,600-bp full-length yeast topoisomerase I gene with URA3 as a selectable marker. To generate pHT-100-K532A, the 859-bp SpH/Nhel fragment of pHT-100 was replaced with the corresponding fragment from pFastBac1-topo70K532A. pHT-100 was cut with BamHI to release the entire topoisomerase I sequence.
on a 3.4-kb fragment and religated to generate the empty vector pHT-100-dTopo1.

The _S. cerevisiae_ strains used in this study are the haploid WT strain W303-1a (MATa ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1) and the topI null mutant RS190 which is identical to W303-1a plus top1-8::LEU2 (28). Both strains were generously provided by R. Starkl. The two _S. cerevisiae_ strains were each transformed with plasmids pHT-100-dTopo1, pHT-100, and pHT-100-K532A using the lithium acetate method (29). Rich (YPD) and minimal (SD) media were prepared as described (30).

For drop assays, two colonies from each transformation were grown overnight at 30 °C in SD-ura medium with glucose as the carbon source. Cultures were then diluted to an _A_ _sub_ of 1, and 5 µl from each of 10-fold dilutions (10^6 to 10^-3) were spotted on solid SD-ura medium. The plates contained either galactose or glucose as carbon source to activate or repress expression of topoisomerase I from the GAL1 promoter, respectively. Plates were incubated at 30 °C, and pictures were taken every day for 4 days.

Sequence Specificity—Two different DNA fragments were used to determine the cleavage specificity of different topoisomerase I variants. Plasmid pGEM-3Zf(-) (Promega) was digested with AarII and NdeI, and the 249-bp fragment was gel-purified. The NdeI end was labeled by a fill-in reaction using T4 DNA polymerase and _[^32]P_dATP. After heat inactivation of the polymerase, the fragment was cleaved with BspHII to leave a 233-bp 3' end-labeled fragment. Following purification by phenol/chloroform extraction and ethanol precipitation, the substrate was resuspended in TE (10 mM tris-HCl, pH 8, 1 mM EDTA).

Reactions (final volume 10 µl) contained 50 mM tris-HCl, pH 7.5, 10 mM MgCl_2, 0.8 ng of WT topo70 and 200 ng each of K532R and K532A protein in complex with a 22-mer duplex DNA oligonucleotide used in previous structural studies (6) were grown by vapor diffusion at room temperature. The drops, containing equal volumes of 48 mM K532R/Y723F-DNA solution and the reservoir (15% PEG 3350 monomethyl ether, 200 mM MgCl_2, 100 mM MES, pH 7.5, and 2 mM TCEP [tris(2-carboxyethyl) phosphine], yielded crystals within 1 week. Crystals grew to ~100 × 100 × 30 µm and were flash-frozen in a cryoprotectant of artificial mother liquor supplemented with 15% ethylene glycol.

**Structure Determination—**Data were collected on beamline 19-BM (Advanced Photon Source, Argonne National Laboratories, Argonne, IL) and processed with HKL2000 (34). Initial phases for the topo70 K532R/Y723F-DNA complex were obtained using molecular replacement methods in AMoRe (35) with the coordinates of human top70 Y723F in a non-covalent complex with a 22-bp DNA duplex oligonucleotide (Protein Data Bank code 1A36) (6) as a search model. Further refinement and model building were performed with alternate rounds of REFMAC5 (36) and Xtalview (37).

**RESULTS**

Expression and Purification of Mutant Proteins—To determine the biochemical importance of Lys_532 in catalysis and sequence selectivity by human topoisomerase I, two mutations were introduced into a 70-kDa form of the WT enzyme (top70). Topo70 lacks the first 174 NH2-terminal amino acids but retains full enzymatic activity _in vitro_ (26). Lys_532 was replaced by alanine and more conservatively by arginine; the mutant proteins will be referred to as K532A and K532R, respectively. The mutant enzymes were expressed in insect cells infected with the empty vector pHT-100-dTopo1.

The mutant enzymes were expressed in insect cells infected with the empty vector pHT-100-dTopo1.

**Fig. 1.** Coomassie-stained SDS-PAGE of purified WT and mutant _topo70_ proteins. The 1st lane contains the molecular weight markers with the molecular mass in kDa indicated on the left. Approximately 2 µg of the indicated proteins were loaded in each lane.

**Fig. 2.** Plasmid relaxation assays. _A_, serial 2-fold dilutions of WT top70, K532R, and K532A enzymes were incubated with 0.5 µg of supercoiled plasmid DNA for 10 min and analyzed by agarose gel electrophoresis. _B_, 0.8 ng of WT top70 and 200 ng each of K532R and K532A were incubated with 0.5 µg of supercoiled plasmid DNA for the indicated times and analyzed by agarose gel electrophoresis. The samples were treated with proteinase K prior to the electrophoresis. The band that migrates slower than the nicked circular DNA consists of dimers of the supercoiled plasmid DNA that also become relaxed.
Effects of Lys532 Mutations on Relaxation of Supercoiled DNA—The effect of the introduced mutations on catalysis was first examined by assaying the ability of the purified proteins to relax a supercoiled plasmid substrate (Fig. 2). Serial 2-fold dilutions of the WT and mutant proteins were incubated with 0.5 μg of supercoiled plasmid DNA. The K532R enzyme was about 100-fold less active than the WT enzyme (Fig. 2A), but when present at ~200-fold higher concentration than the WT enzyme exhibited similar reaction kinetics in a time course experiment under otherwise identical conditions (Fig. 2B). The K532A enzyme was at least 500 times less active than the WT enzyme based on the finding that activity is barely detectable even at the highest protein concentration used in the dilution series experiment (Fig. 2A). After prolonged incubation at a high protein concentration, K532A primarily produced nicked circular DNA (Fig. 2B), with very few relaxed molecules. Because the products of the reaction were treated with proteinase K prior to the gel analysis, an increase in nicked circles (containing a proteinase K-resistant peptide) reflects the accumulation of covalent complexes. Thus, the simplest explanation for this observation is that in addition to the overall low relaxing activity, the cleavage religation equilibrium for K532A is markedly shifted toward cleavage.

Equilibrium Cleavage Assays—To characterize further the mutant enzymes, the 25-mer duplex oligonucleotide substrate CL25/CP25 (Fig. 3A) containing a preferred topoisomerase I cleavage site (Fig. 3A, arrow labeled 1) was used in an SDS cleavage assay. A secondary cleavage site is also present on this oligonucleotide (Fig. 3, arrow labeled 2). This substrate is identical to the 22-bp DNA substrate that was used to obtain the crystal structure of topoisomerase I bound to DNA (5, 6), but contains two additional base pairs at its 5′-end and one additional base pair at the 3′-end. WT topoisomerase I cleaved the 5′-32P-end-labeled CL25 strand in the substrate at both cleavage sites (Fig. 3B, lanes 2–4), whereas cleavage by the two mutant enzymes is detectable primarily at the preferred cleavage site. Because both cleavage and religation occur with this oligonucleotide substrate, the reaction is expected to eventually reach equilibrium. K532R showed 3–5 times less cleavage product at equilibrium than the WT enzyme (Fig. 3B, compare lanes 2–4 with lanes 5–7, and see Fig. 3C) indicating that the cleavage religation equilibrium for this mutant is shifted toward religation. Together with the finding that the overall plasmid relaxation rate of K532R was at least 100 times slower than that of the WT enzyme, we hypothesize that the K532R mutation slows cleavage to a greater extent than religation, resulting in less cleaved product at any given time. The K532A enzyme continued to accumulate cleaved product over the course of the experiment and had not reached equilibrium by 8 h (Fig. 3C). This result confirms that religation is impacted to a greater extent than cleavage for this mutant, and therefore the cleavage-religation equilibrium is shifted toward cleavage. The observed differences in activity between the WT and mutant enzymes are not due to differences in the DNA binding affinity for this substrate as determined in gel shift assays (data not shown).

Suicide Cleavage Assays—The 14-mer DNA oligonucleotide CL14 was 5′-32P-end-labeled and annealed to the complementary oligonucleotide CP25 (Fig. 4A). After cleavage and covalent attachment of topoisomerase I to the 5′-end-labeled CL14, a dinucleotide (cleavage site 1) or tetranucleotide (cleavage site 2) 3′ of the cleavage site unpairs and dissociates, preventing religation. Cleavage reaction mixtures were incubated at 23 °C for up to 22.5 h, and samples were stopped with SDS at the indicated time points. A portion of each sample was analyzed by SDS-PAGE to visualize protein that contained the covalently bound radioactively labeled cleavage product (Fig. 4B). The remaining portion of each sample was digested with trypsin and run on a sequencing gel. The sequencing gel analysis shows that all three enzymes cleaved the oligonucleotide at both sites (Fig. 4D). Because the SDS-PAGE reflects cleavage at both sites, the results of this analysis were used for quantitation of the covalent complexes formed, and the data are presented in Fig. 4C. The WT enzyme reached the maximum amount of covalent complex after about 15 min. Neither mutant enzyme reached a plateau within the 22.5-h incubation; therefore, the amounts of covalent complex formed by the mutant enzymes were normalized to the maximum amount of complex formed by the WT enzyme under the assumption that the mutant enzymes would eventually reach the same plateau.
value. K532R cleaved the suicide substrate at a rate that is 3
orders of magnitudes slower than that of WT topoisomerase I,
whereas the K532A suicide cleavage rate is 50 times slower
than that for K532R.

It is interesting to note that cleavage of this suicide substrate
is substantially slower than cleavage of the similar but fully
duplexed equilibrium cleavage substrate CL25/CP25 (Fig. 3).
With the duplex substrate, WT topo70 had essentially reached
equilibrium by 7 s, the earliest time point taken (Fig. 3C),
whereas it took 15 min to reach a plateau for cleavage of the
suicide substrate (Fig. 4). The presence of single-stranded
rather than duplex DNA downstream of the cleavage site may
be partly responsible for this phenomenon.

Lys532 Acts as a General Acid Catalyst during Cleavage—By
using 5'-bridging phosphorothiolate oligonucleotide substrates
(22), it has been demonstrated recently that Lys167 in vaccinia
topoisoamerase acts as a general acid in the catalysis of the
cleavage reaction. To test whether the equivalent Lys532 in the
human topoisomerase I protein serves the same function, the
5'-bridging phosphorothiolate DNA oligonucleotide CL22-S/
CP22 (Fig. 5A) was used. This substrate acts as a different kind
of suicide substrate than the one used earlier (Fig. 4A). After
cleavage by topoisomerase I, the sulfhydryl group on the 5'-end
of the scissile strand is unable to attack the covalent bond
between the topoisomerase I active site tyrosine and the 3'-end
of the scissile strand of the DNA, thus trapping the topoisomer-
ase I on the DNA (38). In addition, the modification of the
scissile phosphodiester alleviates the need for a general acid to
protonate the leaving 5'-oxygen in the cleavage reaction be-
cause a leaving 5'-sulfur has a much lower pKₐ than the 5'-'oxygen
that would normally be present.

Indeed, DNA cleavage by K532A and K532R was greatly
stimulated by the 5'-bridging phosphorothiolate, and both mu-
tants cleaved the modified substrate at nearly identical rates,
and only somewhat slower than the WT enzyme (Fig. 5B). This
result is in clear contrast to the single turnover data using the
unmodified suicide substrate CL14/CP25 where cleavage by
the mutant enzymes was 3 to 4 orders of magnitude slower
than cleavage of the same substrate by the WT enzyme (Fig.
4C). These data indicate that Lys532 acts as a general acid to
protonate the leaving 5'-oxygen in the human topoisomerase I
cleavage reaction.

Religation Kinetics—To examine directly the religation ca-
pabilities of the mutant K532R and K532A topoisomerase I

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**Fig. 4. Suicide cleavage assays.** A, the structure of the 5'-32P-end-labeled CL14/CP25 oligonucleotide suicide substrate is shown with the arrows indicating the same two cleavage sites as in Fig. 3A. B, the SDS-PAGE shows the suicide cleavage time course. Note that the time course for the WT enzyme is in minutes, while the gels showing suicide cleavage by the K532R and K532A mutant enzymes is given in hours. C, a graphic representation of the suicide cleavage results shown in B. Note that the abscissa for the WT enzyme (inset) is in minutes, while the abscissa for the mutant enzymes is in hours. D, sequencing gel analysis for samples taken during the time course experiment shown in B. Note the different incubation times. All the samples were run on the same gel, but only a subset of lanes is shown here (−, no protein; K/R, K532R; K/A, K532A).
enzymes, the fully duplexed oligonucleotide substrate CL25/CP25 (Fig. 3A) was used. WT and K532R were incubated for 10 min to ensure they reached cleavage equilibrium. K532A was incubated for 130 min to accumulate sufficient covalent complex for easy visualization by SDS-PAGE. At this point, NaCl was added to a final concentration of 500 mM to dissociate the covalent protein DNA complex (40). To test this hypothesis, NaCl was added to 0.5M, and the amount of cleaved complex was quantified after analyzing the product by SDS-PAGE using the PhosphorImager. The data shown here are representative of a total of three experiments.

It is interesting to note that in a fraction of the cases where the protein is covalently attached to the oligonucleotide, no religation was observed (~20% for WT and ~8% for K532R). This is most probably caused by cleavages on the non-labeled strand. WT topoisomerase I cleaves at two sites ~7 to 8 bases from the 5'-end of the unlabeled CP25 (see Fig. 3A and data not shown), thus creating a suicide substrate for subsequent topoisomerase I cleavage on the labeled strand.

The K532A Mutation Has a Deleterious Effect on Yeast Cell Growth That Mimics the Action of CPT—Because K532A was able to cleave the substrate DNA but religation was substantially slowed, we suspected that the mutant enzyme might mimic the effects of CPT, which prolongs the lifetime of the cleavage site which is defined as occurring between the −1 and +1 positions (12, 13). Lys 532 is the only residue in the cleavage site that has been shown to be a valuable tool for the study of yeast and human topoisomerase I and particularly their interactions with antitumor drugs (28, 41–43). A full-length topoisomerase I K532A mutant was cloned into a CEN/ARS yeast E. coli shuttle vector resulting in plasmid pHT-100-K532A, and then either WT human topoisomerase I or the K532A mutant enzyme was expressed from the inducible GAL1–10 promoter in cells deleted for the top1 gene and in WT cells. The top1 deletion mutant yeast strain was hypersensitive to K532A mutant overexpression (Fig. 7), whereas overexpression of WT human topoisomerase I only had a minor effect on cell survival. Only the data derived from expression experiments in the top1 deletion strain are shown because they are virtually indistinguishable from those in WT cells (data not shown). We conclude that human topoisomerase I K532A expression in S. cerevisiae has a dominant-negative effect on cell growth.

Lys32 Is Not Essential for the Weak Sequence Preference of Topoisomerase I—Eukaryotic topoisomerase I has a weak sequence preference for the bases at positions −4 to −1 relative to the cleavage site which is defined as occurring between the −1 and +1 positions (12, 13). Lys32 is the only residue in the crystal structure of topoisomerase I that makes a base-specific contact to the substrate DNA (5). This contact is in the minor groove and involves the O-2 carbonyl oxygen of the −1 thymine

![Image 5](https://example.com/image5.png)

**Fig. 5.** Suicide cleavage using a 5'-bridging phosphorothiolate containing DNA oligonucleotide. A, the structure of the 5'-32P-end-labeled CL22-S/CP22 oligonucleotide substrate containing a 5'-bridging phosphorothiolate linkage (−OPS−) is indicated with the cleavage site marked with an arrow. B, WT and the K532A and K532R mutant enzymes were incubated with the suicide substrate CL22-S/CP22 for the indicated times, and the reactions were stopped with SDS. The amount of covalent complex was quantified after analyzing the product by SDS-PAGE using the PhosphorImager. The data shown here are representative of a total of three experiments.

![Image 6](https://example.com/image6.png)

**Fig. 6.** Religation assays. A, the 5'-32P-end-labeled CL25/CP25 oligonucleotide substrate described in Fig. 3A was incubated with the WT and mutant enzymes to allow cleavage. To monitor the rate of religation, NaCl was added to 0.5 M, and the amount of cleaved complex remaining was quantified by SDS-PAGE. B, the kinetics of religation are plotted for the WT and mutant enzymes. (K532A is shown in inset.) The amount of covalent complex is normalized to the amount of complex present at the 0 time point when the salt was added. Duplicate religation experiments with identical time points, including the one shown in A, were averaged for each data point, and the error bars indicate the ±S.D. of the individual values from the mean. Three additional similar experiments support the data shown.
base in the scissile strand, the base that is strongly preferred at this position.

This finding led us to hypothesize that this interaction might be important for the sequence preference for a T at this position. To test this hypothesis, a 3'-end-labeled 233-bp DNA fragment was incubated with 100 ng of the topoisomerase I enzymes indicated in Fig. 8, and the reactions were stopped with SDS. The WT enzyme was also used at the lower amount of 25 ng per reaction (Fig. 8, lanes 3 and 9) to achieve a total amount of cleavage that was more comparable with the mutant enzymes. CPT, which enhances cleavage, was included in a parallel series of reactions to better visualize the cleaved products. Although the relative cleavage efficiencies differ when comparing the mutant enzymes with the WT, overall it can be seen that both mutant enzymes cleaved only at positions also cleaved by the WT enzyme. This is particularly easy to see in the CPT-treated samples (lanes 8–12), but most of the same bands are also visible in lanes 2–6 where CPT was omitted from the reactions. Only seven sites that are cleaved by both mutant enzymes and the WT enzyme could be precisely mapped from the results shown in Fig. 8 (GCAT/C, GGCT/T, GTGT/C, GTTG/G, TGTT/G, GTGT/T, and GTCA/G). Five additional sites could be mapped on a different 141-bp fragment (TCTT/T, CAAT/A, CCAT/G, TTTTA, and ACTTA, data not shown).

An additional interesting observation was made in this experiment. The K532A mutant accumulates cleaved product over time as seen before in the equilibrium cleavage assay. Furthermore, this mutant enzyme is resistant to CPT because addition of the drug does not cause an increased amount of cleaved product (compare lanes 5 and 11 and lanes 6 and 12, respectively). The simplest interpretation of this observation is that the K532A enzyme relatiges so slowly that the drug does not have any additional effect. This is in agreement with the observation that religation by K532A is much slower than religation by the WT enzyme.

Comparison of the topo70 K532R/Y723F Structure with the topo70 Y723F Structure—To better understand the remaining catalytic activity of K532R, the more active of the two mutant proteins, we combined the K532R mutation with the active site mutation Y723F that renders the topoisomerase completely inactive. This second mutation enabled the resulting topo70 K532R/Y723F mutant protein to be crystallized in a non-covalent complex with duplex DNA (5). The 3.1 Å data set for the topo70 K532R/Y723F protein showed clear electron density for 549 amino acids extending from 202–633, 644–713, and 719–765 with 29 ordered water molecules. Statistics are shown in Table I. The overall structure of the topo70 Y723F/K532R protein in complex with a 22-bp DNA is very similar to the topo70 Y723F structure, with a root mean square deviation of 0.93 Å over 549 Ca atoms.

![Fig. 7. Expression of human topoisomerase I K532A in S. cerevisiae.](image1)

**Fig. 7. Expression of human topoisomerase I K532A in S. cerevisiae.** 5 μl of 10-fold serial dilutions (indicated by triangles of top1 mutant yeast cells containing either empty vector or plasmids encoding WT human topoisomerase I or the K532A mutant were spotted on plates in duplicate. Expression of the proteins is repressed in glucose-containing medium and induced in the presence of galactose.

![Fig. 8.](image2)

**Fig. 8. Sequence specificity of WT and mutant enzymes.** WT topoisomerase I, K532R (K/R), and K532A (K/A) were incubated with a 3'-end-labeled 233-bp DNA fragment in the absence (lanes 2–6) or presence (lanes 8–12) of 50 μM CPT. 100 ng of the respective enzymes were used per reaction, except in lanes 3 and 9 where only 25 ng of the WT enzyme were used. Incubation time was 30 min for all reactions, except for the reactions loaded in lanes 6 and 12 which were incubated for 4 h. In lanes 1 and 7 the substrate DNA alone was loaded, without and with CPT, respectively. Lane 13 contains an A + G ladder, and lane 14 contains a T ladder. Reactions were analyzed on a 6% sequencing gel.

### Table I

| Data collection and refinement statistics |  |
|-----------------------------------------|--|
| **Data collection statistics** |  |
| Cell dimensions Å× | a = 56.9, b = 118.6, c = 71.7, β = 98.3° |
| Resolution Å | 3.1 |
| Unique reflections | 15497 |
| Redundancy | 3.2 (1.7) |
| Completeness % | 93.8 (64.7) |
| Rsym % | 14.5 (28.0) |
| Average I/σ | 8.9 (3.3) |
| **Refinement statistics** |  |
| Rcryst % | 27.7 |
| Rfree % | 33.9 |
| Figure of merit % | 71.9 |
| r.m.s.d.° bonds Å | 0.004 |
| r.m.s.d.° angles ° | 0.763 |
| No. residues | 549 |

* r.m.s.d., root mean square deviation.

Despite the similarities in the overall structure, three notable differences were found within the active site of the double mutant protein when compared with topo70 Y723F. First, the Arg532 is able to maintain the base-specific contact to the O-2 of the −1 thymine base, with only −3 Å between the side chain amines of the arginine side chain and the O-2 atom (Fig. 9). The slightly longer chain of arginine versus lysine, however, induces a 1.20 Å shift in the position of the scissile phosphate when compared with topo70 Y723F. Second, with this shift of the phosphate, Arg532 is no longer within hydrogen bonding
distance of the non-bridging oxygen of the scissile phosphate, as both terminal amines are now greater than 4 Å away (Fig. 9). The third major change occurs in the loop region following helix 17. The helix leading up to the residue 633 shows a significant shift in the Cα positions (greater than 3 Å) from topo70 Y723F protein. Particularly interesting is the shift in position of the side chain of His632, which now points away from the DNA entirely and is unavailable to form a hydrogen bond to a non-bridging oxygen of the scissile phosphate. Additionally, the next residue in the chain, Gln633, points its side chain to where the imidazole of His632 normally resides. Although the side chain of Gln633 is too far away (4.5 Å) to hydrogen-bond to the non-bridging oxygen of the scissile phosphate, it is oriented toward the phosphate in the active site (Fig. 9).

**DISCUSSION**

Based on biochemical and structural data obtained from human topoisomerase I, vaccinia topoisomerase, and tyrosine recombinases, four amino acid residues have been implicated in catalysis by type IB topoisomerases and tyrosine recombinases (1, 5, 14, 15). For the human enzyme these residues are Arg590, Arg599, His632, and the nucleophilic Tyr723. The data presented here firmly establish Lys532 as a fifth catalytic residue of human topoisomerase I, similar to Lys172 of XerD, Lys823 of Flp, and Lys167 of vaccinia topoisomerase I. Mutating these lysines to alanine nearly abolishes catalytic activity of the respective enzymes, whereas the more conservative change to arginine, whenever analyzed, causes a less drastic reduction in activity (19–21).

The position of Lys532 relative to the scissile phosphate and other active site residues in topoisomerase I crystal structures provides key insights into the normal function of this amino acid in catalysis. In the crystal structure with a bound 22-bp oligonucleotide containing a thymine at the −1 position (−1T), Lys532 is hydrogen-bonded to the O-2 atom of the thymine base but does not otherwise contact the DNA (5). However, in the crystal structure containing the same oligonucleotide sequence except for a cytosine instead of a thymine at the −1 position (−1C), Lys532 is hydrogen-bonded to both the O-2 atom of cytosine and one of the non-bridging oxygens of the scissile phosphate (14). Interestingly, Lys823 of Flp also contacts the −1 base and a non-bridging oxygen (44). For human topoisomerase I, we have argued previously (14) that the −1C structure may represent a more advanced stage along the catalytic pathway than the configuration found in the −1T structure. If so, these observations implicate the positive charge on the side chain of Lys532 in the stabilization of the pentavalent transition state during cleavage. The other major difference between the two structures that is consistent with this conjecture is that Arg590 shifts from a position where it makes a hydrogen-bonding contact with a non-bridging oxygen of the scissile phosphate in the −1T structure into close proximity with the hydroxyl of Tyr723 in the −1C structure, where it could act to stabilize the phenolate anion of the attacking nucleophile.

Our functional analyses of mutations in human topoisomerase I at the Lys532 locus indicate that in addition to transition state stabilization, Lys532 also serves as a general acid catalyst during the cleavage reaction. This conclusion is based on the use of the 5′-bridging phosphorothiolate-containing substrate for which the 5′-SH is a much better leaving group than the normal 5′-OH during cleavage and obviates the need for a general acid to protonate the leaving atom. The rate of cleavage for enzymes containing either an alanine or an arginine in place of lysine at position 532 is several orders of magnitude faster than with the normal unmodified substrate. This is the expected result if Lys532 normally acts as a general acid to protonate the leaving 5′-oxygen during cleavage.

Does Lys532 also contribute to the sequence preference of human topoisomerase I? In particular, based on the available crystal structures, it seemed possible that the contact with the O-2 atom of the −1 base could be responsible for the preference for a thymine or cytosine residue at the −1 position. Because neither K532A nor K532R shows a changed cleavage pattern compared with WT (Fig. 8), we can exclude this possibility. In fact, in all of the co-crystal structures of tyrosine recombinases containing bound DNA, the lysine residues corresponding to Lys532 of human topoisomerase I make base-specific contacts similar to the one observed in the human topoisomerase (44–46). Although these enzymes bind the DNA in a sequence-specific manner, this specificity seems to be principally determined by additional base-specific interactions and not by the contact to the lysine (45, 47–50). Taken together, these data suggest that the contact between Lys532 and the −1 base, and the respective interactions in the tyrosine recombinases, are directly involved in the cleavage and religation reactions. Although the exact role for these lysine-DNA contacts is unclear, we hypothesize that these interactions contribute to catalysis by providing spatial rigidity to the active site region of the enzyme. This feature of the active site may be particularly important for positionally constraining the 3′-end of the cleaved strand during religation.

Given a role for Lys532 in transition state stabilization and as a general acid catalyst, it is not surprising that substituting...
alanine at this position reduces the activity of the enzyme by all measures to near-background levels. In principle, arginine at this position could act to stabilize the transition state by contacting a non-bridging oxygen of the scissile phosphate, but the crystal structure of K532R suggests that the longer arginine side chain, while still contacting the O-2 atom of the base, could play this role effectively only if the architecture of the active site is modified during catalysis as described above for the 1C crystal structure. In addition, arginine at position 522 with its basic side chain could still serve as a general acid, albeit a weak one, due to the difference in pKa between the lysine and arginine side chains. Thus, it would appear that some of the functionality attributed to Lys532 is retained by arginine at this position, accounting for a higher residual level of activity for K532R as compared with K532A.

Although both the K532R and K532A mutant enzymes retain but a small fraction of the WT level of relaxing activity, it is clear that the mutations have different effects on the cleavage and religation components of the reaction. Based on measurements of the cleavage recombination equilibrium, replacing Lys532 with an arginine has a relatively greater impact on cleavage than on religation, whereas the reverse is true when Lys532 is replaced with an alanine. A direct comparison of the recombination rates for the two mutants (Fig. 6B) confirms the inability of K532A to catalyze religation at a significant rate and explains the accumulation of covalent complexes during the reaction. The ability of the K532R mutant to catalyze religation is likely related to the interaction of the arginine side chain with the –1 base as revealed by the crystal structure and a residual ability to stabilize the transition state. Interestingly, both mutant enzymes cleave the 5′-bridging phosphorothiolate oligonucleotide substrate at the same rate, whereas K532A is much more impaired than K532R in the cleavage of unmodified oligonucleotide. Because the 5′-bridging phosphorothiolate eliminates the need for a general acid in the cleavage reaction, this observation suggests that the difference between the K532R and K532A mutants in the cleavage reaction on the normal substrate is the residual ability of the arginine residue to function as a general acid. The inability of K532A to religate the DNA after cleavage makes it a so-called "toxic" topoisomerase. The term toxic is applied to these enzymes because of their reduced ability to religate the DNA, which leads to a trapped covalent complex. In fact, expression of K532A in S. cerevisiae has a deleterious effect on cell growth, presumably because the mutation mimics the effect of the topoisomerase I-specific drug CPT. This effect is similar to what is observed when the S. cerevisiae Top1 proteins that carry mutations in Thr 722 or Arg 517 are expressed in yeast cells (51). Notably, K532A is the first mutant in a catalytic residue that mimics the action of CPT. Mutating Thr716 of human topoisomerase I, the equivalent of yeast Thr722, also mimics the effect of CPT (52), but this effect seems to be of a more structural nature. Based on the crystal structure of human topoisomerase I with the CPT analog topo- tecan, it was suggested that the mutation at Thr716 allows greater mobility of the reactive 5′-hydroxyl on the cleaved strand, thus making religation more difficult (53).

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