The Role of Histidine 632 in Catalysis by Human Topoisomerase I*

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Based on the crystal structure of human topoisomerase I, we hypothesized that hydrogen bonding between the side chain of the highly conserved His632 and one of the nonbridging oxygens of the scissile phosphate contributes to catalysis by stabilizing the transition state. This hypothesis has been tested by examining the effects of changing His632 to glutamine, asparagine, alanine, and tryptophan. The change to glutamine reduced both the relaxation activity and single-turnover cleavage activity by approximately 100-fold, whereas the same change at three other conserved histidines (positions 222, 367, and 406) had no significant effect on the relaxation activity. The properties of the mutant protein containing asparagine instead of histidine at position 632 were similar to those of the glutamine mutant, whereas mutations to alanine or tryptophan reduced the activity by approximately 4 orders of magnitude. The reduction in activity for the mutants was not due to alterations in substrate binding affinities or changes in the cleavage specificities of the proteins. The above results for the glutamine mutation in conjunction with the similar effects of pH on the wild type and the H632Q mutant enzyme rule out the possibility that His632 acts as a general acid to protonate the leaving 5'-oxygen during the cleavage reaction. Taken together, these data strongly support the hypothesis that the only role for His632 is to stabilize the pentavalent transition state through hydrogen bonding to one of the nonbridging oxygens.

Type I topoisomerases promote the relaxation of supercoiled DNA by introducing a temporary break in one of the strands of the helix (1). This function is important for a number of different cellular processes such as DNA replication, transcription, and chromatin remodeling (1, 2). The type I topoisomerases have been divided into two subfamilies, type IA and type IB. These two subfamilies share no sequence homology and relax DNA by distinct mechanisms. The type IA subfamily was originally identified in prokaryotes but is now known to include topoisomerases III from eukaryotic organisms as well (3, 4). *Escherichia coli* topoisomerase I is the prototype of the type IA subfamily (1). Type IA topoisomerases relax only negatively supercoiled DNA and require a region of single-stranded DNA as well as Mg2+ for DNA relaxation.

The type IB topoisomerase subfamily includes eukaryotic topoisomerase I reactions traps the enzyme molecules at the nicked intermediate stage (2). This technique has facilitated the mapping of break sites on duplex DNA fragments (9). A large number of SDS-induced break sites have been characterized that define a weak consensus sequence for cleavage of 5'-A or T-G, C, or A/A or T-T-3' in which the enzyme is covalently attached to the 3'-most thymidine nucleotide (defined as the -1 residue) (9–13). A strong break site has been identified within a 30-bp repeated hexadecameric sequence derived from the rDNA spacers of *Tetrahymena thermophila*, which has been shown to be efficiently cleaved by all eukaryotic type I topoisomerases examined (14, 15).

Human topoisomerase I is composed of 765 residues with a molecular mass of 91 kDa (16). Sequence comparisons of cellular type IB eukaryotic topoisomerases and limited proteolysis studies combined with the crystal structure of the enzyme (5, 8, 17) have defined four major domains as follows: an NH₂-terminal domain (Met1–Gly214), a core domain (Ile215–Ala635), a linker domain (Pro636–Lys712), and a COOH-terminal domain (Gln713–Phe765). The NH₂-terminal domain is poorly conserved, highly charged and unstructured, and dispensable for activity in vitro (8, 17). It contains four putative nuclear localization signals and has been shown to interact with nucleolin (19) and perhaps other nuclear proteins as well (20). Topo701 is a truncated form of human topoisomerase I with a molecular mass of 70 kDa that lacks residues 1–174 of the NH₂-terminal domain and retains full enzyme activity in vitro (8, 21). The core domain is highly conserved and more protease-resistant.

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1 The abbreviations used are: topo70, NH₂-terminal truncation of human topoisomerase I missing the first 174 amino acids; WT, the indicated form of the human topoisomerase I enzyme containing the wild type amino acid sequence; DTT, dithiothreitol; CPT, camptothecin, bp, base pair(s); Mes, 4-morpholineethanesulfonic acid; GST, glutathione S-transferase.
than the other domains. The crystal structure of human topoisomerase I indicates that the core domain can be further divided into three subdomains as follows: subdomains I and II that fold together to form the cap structure that covers one side of the DNA, and subdomain III that contains all of the active site residues with the exception of Tyr723 and that buries the DNA on the side opposite the cap (18). The linker domain forms a coiled-coil structure that protrudes from the body of the enzyme and connects the core to the highly conserved COOH-terminal domain (5). The active site tyrosine (Tyr723) is located in the COOH-terminal domain close to the scissile phosphate in the bound DNA (18). Complete enzymatic activity can be reconstituted by mixing a core fragment with a COOH-terminal domain fragment (22), thus the linker region is dispensable for activity in vitro.

Although the details of catalysis for the eukaryotic topoisomerase I reaction still remain to be elucidated, based on the crystal structure of the non-covalent human topoisomerase I-DNA complexes (5, 23, 24), both Arg488 and Lys532 appear to function as a general acid-base catalyst, we describe here the properties of topo70 have been described previously (8). The purification of the mutant forms of topo70 have been described previously (8). The procedures for baculovirus infection and purification of WT topo70 have been described previously (8). All mutations were confirmed by DNA sequencing of the recombinant baculovirus stocks. The procedures for baculovirus infection and purification of WT topo70 have been described previously (8). The purification of the mutant proteins was similar except that the topo70 H632N and topo70 H632A proteins eluted from the Mono S column at a slightly higher KCl concentration than WT topo70 (160 mM instead of 140 mM). The purified proteins were stored in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, and 50 μg/ml bovine serum albumin. The substrate used for the relaxation assays was pBluescript KSII+ plasmid DNA (Stratagene) (50 ng/μl). For the serial dilution, the enzymes were 2-fold serially diluted in Reactivation Buffer yielding final concentrations of 0.25 ng/μl. For the gel shift assays, the enzyme concentrations were carried out at an enzyme concentration of 0.25 ng/μl in a final volume of 20 μl. The indicated times, 20-μl aliquots were removed, and the reaction was stopped by the addition of 5 μl of Stop Buffer (2.5% SDS, 25 mM EDTA, 25% Ficoll 400, 0.08% bromphenol blue, 0.08% xylene cyanol). For the serial dilution assay, the enzymes were 2-fold serially diluted in Reaction Buffer, and the reactions were initiated by the addition of 2 μl of the diluted enzyme to 18 μl of Reaction Buffer containing DNA. The reactions were incubated at 37 °C for 30 min and carried out at an enzyme concentration of 0.25 μl of Stop Buffer. The products were analyzed by electrophoresis in a 0.8% agarose gel and visualized with an UV illuminator after ethidium bromide staining.

**Site-directed Mutagenesis—**Single mutations were introduced into the human topoisomerase I cDNA using the uracil-DNA method described by Kunke et al. (28) or by using the two-stage megaprimer PCR method (29). To make the H222Q, H367Q, and H427Q mutations, the Xhol/HVeI fragment of human topoisomerase I was cloned in the pBluescript KS+ vector and transformed into E. coli CIJ236 (der ‘ung’) to obtain the uracil-containing single-stranded DNA. After introducing the mutations, the topo70 coding region (8) with or without the mutations was cloned in the pGEX vector for GST fusion protein in E. coli. For the H632Q mutation, three oligonucleotide primers were synthesized for use in the megaprimer PCR method as follows: two flanking primers with restriction sites for future cloning and a megaprimer with the single mutation. One of the flanking primers and the megaprimer were used for the first stage PCR. The first stage PCR product as well as the other flanking primer were used for the second stage PCR. The second stage PCR product containing the H632Q mutation was cloned in the pGEX-topo70 vector for GST fusion protein expression and purification from E. coli.

A three-way ligation was used to generate the recombinant pFASTBAC1 plasmid (Life Technologies, Inc.) containing the topo70 coding region with the H632Q mutation. The pFASTBAC1 vector was cut with BamHI and EcoRI, and the large fragment was combined with the HindIII-EcoRI fragment from pGEX-topo70-H632Q (above) containing the mutation and the BamHI-HindIII fragment from pDH1Btopo70 (30). After ligation and transformation of the desired pFASTBAC1 recombinant plasmid (pFASTBAC1 topo70-H632Q), the Bac-to-Bac system (Life Technologies, Inc.) was used to generate the recombinant baculovirus expressing the topo70 H632Q protein according to the protocol provided by the manufacturer. The PCR-based megaprimer method (29) was used to generate PpuMI-NH2 topoisoamerase I-derived fragments containing the H632A, H632N, and H632W mutations. These fragments were used to replace the corresponding fragment in pFASTBAC1 topo70 H632Q to yield the additional mutant recombinant pFASTBAC1 derivatives. The generation of the recombinant baculovirus expressing WT topo70 was described previously (8). All mutations were confirmed by dye-exchange sequencing of the region derived from the PCR fragment.

**Gel Shift Assay—**A 25-mer DNA oligonucleotide (CL25, see Fig. 4) was end-labeled by phosphorylation with T4 polynucleotide kinase in the presence of [γ-32P]ATP (3000 Ci/mmol). To prepare the duplex substrate, the labeled oligonucleotide was annealed to a 2-fold molar excess of the complementary 25-mer (CP25, Fig. 4) by heating to 94 °C for 1 min, followed by 10 min at 65 °C and then cooling to 25 °C for another 10 min. The DNA binding assay was carried out in 10 μl of Reaction Buffer containing the labeled duplex oligonucleotide (0.5 nM) and 2-fold serial dilutions of the topoisomerase yielding final concentrations ranging from 10 nM to 0.064 μM. The reactions were incubated at room temperature for 15 min followed by the addition of 2.5 μl of 50% glycerol and analyzed by electrophoresis in a nondenaturing 6% polyacrylamide gel at 4 °C. The running buffer contained 25 mM Tris-HCl and 162 mM glycine, pH 8.5. Due to the high pI value of the topo70 protein (9.3), free protein and protein-DNA complexes migrated to the cathode under these electrophoresis conditions, and therefore only the unbound DNA entered the gel. The amount of unbound oligonucleotide bound in the gel was quantified by phosphorimagery of the gel image. Quant software (Molecular Dynamics), and the dissociation constant (Kd) was determined from the protein concentration at which one-half of the total oligonucleotide was bound (31).

**Suicide Cleavage Reactions—**A 14-mer DNA oligonucleotide (CL14, Fig. 5) was end-labeled as described above and annealed with a 3-fold molar excess of the complementary CP25 oligonucleotide. The comple-
mentary strand was phosphorylated at the 5' end to prevent self-relation. The two strands were annealed by heating to 94 °C for 1 min followed by slow cooling to 25 °C for 2 h. The single-turnover suicide cleavage reactions were carried out by incubating a 14-fold molar excess of the topoisomerase I protein with the suicide cleavage substrate (20 nm) in Cleavage Buffer (150 mM KCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT) at 23 °C in a final volume of 100 μL. A 10-μl sample of the reaction mixture prior to the addition of protein was removed and used as the zero time point. 10-μl aliquots were removed at the indicated times and mixed with an equal volume of 1% SDS to stop the reaction. Samples were ethanol-precipitated and resuspended in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and digested at 37 °C for 1 h to cleave all but a short peptide of the enzyme from the covalent complex. The digested samples were analyzed by electrophoresis through a 15% polyacrylamide-urea gel (SequaGel from National Diagnostics). Uncleaved oligonucleotide migrates as 14-mer, whereas the cleaved 12-mer oligonucleotide with the attached peptide migrates slightly slower. The extent of covalent complex formation was quantified using the PhosphorImager and ImageQuant software. The percentage of cleaved oligonucleotide (C%) was determined based on the end point cleavage values. The cleavage rate (k_c) was determined by fitting the data from the first three time points to the equation ln(100 − C%) = 4.605 − k_c.t (26, 32). The rate constant for religation (k_r) was calculated according to the following equation where k_CIC is the equilibrium constant for the cleavage-religation reaction (see below), k_c = k_r/K_CIC. Implicit in this calculation is the assumption that the rate of cleavage with the suicide substrate is the same as the cleavage rate on the completely duplex oligonucleotide. This assumption has been shown to hold true for vaccinia topoisomerase (32).

To determine the effect of pH on the suicide cleavage rate, the Cleavage Buffer was modified using the following buffers (50 mM): pH 6.2, 6.5, 6.9, 8.3, 8.4, 8.9, and 9.4. The percent cleavage was quantified as described above, and the logarithm of the cleavage rate was plotted against the pH.

**Results**

Site-directed Mutagenesis of Conserved Histidine Residues in Human Topoisomerase I—Depending on whether it is protonated or not, a histidine side chain is an ideal candidate to act either as general acid or a general base during the cleavage and religation transesterification reactions catalyzed by human topoisomerase I (34). The solution of the crystal structure of human topoisomerase I provided support for this view as the highly conserved His^{632} was found to be relatively close to the 5'-oxygen of the leaving deoxyribose sugar (5, 18). To test this possibility directly, we used site-directed mutagenesis to change His^{632} to glutamine; like histidine, glutamine can participate in hydrogen bonding, but unlike histidine it is unable to act as a general acid-base catalyst. As controls, we independently changed each of the other three invariant histidines in topoisomerase I proteins (His^{222}, His^{367}, His^{406}) to glutamine as well.

The histidine to glutamine mutations were introduced into the topo70 form of human topoisomerase I, and the mutant proteins were expressed in E. coli as GST fusions. Although human topoisomerase I is unstable when expressed in E. coli, we found previously that sufficient protein is present in crude extracts to permit reliable enzyme assays (35). For these experiments, we purified the four GST fusion proteins using glutathione-Sepharose 4B beads and carried out plasmid relaxation assays using equal amounts of protein as determined by SDS-polyacrylamide gel electrophoresis. The effects of the mutations on enzyme activity were analyzed by a standard serial dilution DNA relaxation assay, and the results are shown in Fig. 1. Only a trace amount of enzyme activity was detected for the H632Q mutant protein when the undiluted sample was used in the assay (Fig. 1, lane 28). However, the activities of the H222Q, H367Q, and H406Q topo70 proteins were nearly indistinguishable from that of WT topo70, demonstrating that the change of a highly conserved histidine residue did not invariably abolish the activity of the enzyme. Apparently, these three amino acids are conserved for reasons having nothing to do with the catalytic activity of the protein. Overall, these results confirm the essential nature of His^{632} in the topoisomerase I reaction as predicted by the crystal structure (5, 18).

**Effects of H632N, H632A, and H632W Mutations on Relaxation of Plasmid DNA**

The cleavage specificities of the WT topo70 (WT, lanes 2–9), H222Q (lanes 10–15), H367Q (lanes 16–21), and H406Q (lanes 22–27) mutant proteins (~5 ng) were 2-fold serially diluted and incubated with 0.5 μg of supercoiled plasmid DNA in Relaxation Buffer for 30 min at 37 °C. A control reaction without added enzyme is shown in lane 1. Since little activity was detected for the H632Q protein even with the undiluted sample, only the assay for the highest protein concentration tested is shown (lane 28). The products of the relaxation reactions were visualized by ethidium bromide staining after agarose gel electrophoresis.

**Fig. 1. Effects of mutating conserved histidine residues on the plasmid relaxation activity of human topoisomerase I.** Equal quantities of WT topo70 (WT, lanes 2–9), H222Q (lanes 10–15), H367Q (lanes 16–21), and H406Q (lanes 22–27) mutant proteins (~5 ng) were 2-fold serially diluted and incubated with 0.5 μg of supercoiled plasmid DNA in Relaxation Buffer for 30 min at 37 °C. A control reaction without added enzyme is shown in lane 1. Since little activity was detected for the H632Q protein even with the undiluted sample, only the assay for the highest protein concentration tested is shown (lane 28). The products of the relaxation reactions were visualized by ethidium bromide staining after agarose gel electrophoresis.
Histidine 632 and Human Topoisomerase I Catalysis

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified WT and mutant proteins with changes at residue 632. The topo70 forms of the indicated proteins were expressed using the baculovirus expression system and purified as described under “Experimental Procedures.” The purified proteins (~4 µg each) were analyzed by electrophoresis in a 10% SDS-polyacrylamide gel. The markers in the leftmost lane had molecular masses of 26.6, 36.5, 48.5, 58, 84, and 116 kDa, respectively.

**Action Activity**—We extended our analysis of the role of His632 by replacing this residue with asparagine, alanine, or tryptophan. The WT and four mutant topo70 proteins (H632Q, H632N, H632A, and H632W) were expressed from recombinant baculoviruses and purified from infected Sf9 insect cells. All of the proteins were stable in insect cells and were purified to near homogeneity (Fig. 2).

The effects of the mutations on enzyme activity were analyzed by standard plasmid DNA relaxation time course assays with a molar ratio of DNA to enzyme of 4:1 (Fig. 3, panel A). WT topo70 completely relaxed the DNA within 2 min under these conditions, whereas all four mutant enzymes exhibited slowed relaxation kinetics. Complete relaxation by the H632Q enzyme was not observed until ~40 min, and thus the H632Q protein appeared to be ~20-fold less active than the WT enzyme. H632N had less activity than H632Q, failing to relax completely the supercoiled DNA even after 40 min. Very little relaxing activity was detectable for the H632A and H632W proteins. We estimate the activity of the H632A and H632W proteins to be more than 100-fold reduced compared with WT topo70.

Supercoiled DNA relaxation under conditions of limiting topoisomerase I is stimulated ~10-fold in the presence of 10 mM Mg²⁺, likely as a result of an increase in the dissociation rate of the enzyme from the DNA (8, 32). It follows that the rate-limiting step for DNA relaxation by the WT topoisomerase I under the normal assay conditions is enzyme dissociation (32). This effect can be seen in Fig. 3 (panel B), where the addition of 10 mM Mg²⁺ to the WT topo70 reaction increased the rate to a value too fast to measure (reaction complete in <5 s). However, the presence of Mg²⁺ in the reactions for the His632 mutant proteins had no effect on the relaxation rates (Fig. 3, panel B), suggesting that enzyme chemistry rather than enzyme dissociation was the rate-limiting step for all of the mutant enzymes. Moreover, in the presence of 10 mM Mg²⁺, the differences between the estimated activity for WT topo70 and the activities of the mutant proteins were magnified: H632Q was at least 100-fold less active than WT topo70, whereas H632N was more than 200-fold less active than the WT enzyme.

**DNA Binding as Measured by Gel Shift Assay**—To test whether the observed reduction in relaxing activity for the mutant proteins resulted from a reduced affinity of the enzymes for DNA, a native gel mobility shift assay was used to compare the DNA binding properties of the mutant proteins with WT topo70. Because topo70 has a pI value of 9.3, the enzyme is positively charged under standard electrophoresis conditions (pH 8.5) and fails to enter the gel. In addition, binding of a negatively charged duplex DNA oligonucleotide (CL25:CP25) (Fig. 4, panel A) only partially neutralizes the charge on the protein and thus the protein-DNA complexes also remain in the well of the gel. Consequently, in this gel shift assay only the unbound duplex oligonucleotide enters the gel. From the reduction in the amount of free oligonucleotide in the gel with increasing concentrations of protein, it was possible to compare the substrate binding properties of the various forms of the protein (Fig. 4, panels B and C). Under these conditions, the protein concentration at which the amount of unbound oligonucleotides was reduced by a factor of 2 is equal to the Kd (31). The binding profiles revealed that the affinity of the mutant proteins for DNA substrate was about the same as that of the WT enzyme with Kd values of ~50 nM (Fig. 4, panel C). These results demonstrated that the reduction in relaxing activity for the mutant proteins did not result from a defect in DNA binding.

**Suicide Cleavage Assay**—A suicide oligonucleotide substrate containing the topoisomerase I cleavage sequence ACTT was used to examine the effect of the mutations on cleavage under single-turnover conditions. The 5’ end-labeled 14-mer scissile strand (CL14) was annealed to the CP25 complementary strand to produce a 14-bp duplex with an 11-base 5’ single-stranded extension (Fig. 5). Upon cleavage and formation of the covalent complex, the dinucleotide AG at the 3’ end of the scissile strand is released, preventing religation. Treatment of the cleavage products with trypsin leaves only a small topoisomerase I-derived peptide covalently attached to the 3’ end of the 12-mer cleavage product. The resulting complex can be resolved from the residual uncleaved oligonucleotide by electrophoresis in a polyacrylamide-urea gel. WT topo70 or the mutant proteins were incubated with the suicide substrate, and the amount of cleaved product (after normalization to the plateau value for the WT enzyme) was plotted against the time of incubation (Fig. 5). Suicide cleavage by the WT enzyme at the earliest time point (5 s) reached more than 20% of the final cleavage value and was complete by ~5 min. Based on the results from three independent analyses, we estimated the cleavage rate (kcat) for WT topo70 to be 0.036 s⁻¹, which is only a factor of 2 slower that the value determined for the vaccinia topoisomerase (32). The cleavage rates for the H632Q and H632N mutant enzymes were approximately 2 orders of magnitude slower than that of the WT enzyme, with kcat values of 3.8 × 10⁻⁴ and 2.0 × 10⁻⁴ s⁻¹, respectively (Fig. 5 and Table I). The cleavage rates for the H632A and H632W proteins were just detectable above the background with estimated kcat values of 6 × 10⁻⁶ and 2.3 × 10⁻⁶ s⁻¹, respectively (Fig. 5 and Table I). Thus, the effects of the various changes at position 632 on the cleavage rates quantitatively parallel the reductions in the rates of relaxation described above.

**Cleavage-Re-ligation Equilibrium**—Since the rates of religation were too fast to obtain reliable measurements, we instead measured the equilibrium cleavage value (KCR) for the WT and the H632Q and H632N mutant topo70 proteins, and we combined these values with the cleavage rates to estimate the rates of religation. We measured KCR using SDS-induced cleavage of the duplex oligonucleotide shown in Fig. 4, panel A, under conditions of excess enzyme and at a substrate concentration such that all of the oligonucleotide should be enzyme-bound (22). To ensure that equilibrium had been reached, two different time points (1 and 2 h) were assayed in each case. The cleavage percentage (%C) of the labeled scissile strand was determined by urea-polyacrylamide gel electrophoresis (Fig. 6) followed by quantitation using the PhosphorImager, and KCR was calculated as described under “Experimental Procedures.”

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The $K_{CR}$ for the H632N mutant was only slightly reduced relative to WT topo70, but a distinct shift toward religation was apparent for the H632Q mutant protein. The magnitudes of the reductions in the $K_{CR}$ values for both mutant proteins as compared with the WT enzyme are less than what one would expect if the only effect of the mutations was to reduce the rates of cleavage. Assuming that the cleavage rates determined with the partially single-stranded suicide substrate ($k_{cl}$) reflect the true cleavage rates on the completely duplex oligonucleotide used for the measurements of $K_{CR}$, the
rates of religation \( (k_r) \) can be calculated using the equation 
\[
K_{CR} = k_r/k_c. 
\]
Although this assumption has not been verified for the human enzyme, it has been shown to hold true for the vaccinia topoisomerase (32). The calculated values of \( k_c \) for WT topo70 and the H632N and H632Q mutant proteins are included in Table I. The extents of cleavage and the cleavage rates for the H632A and H632W proteins were substantially reduced below those of the other mutants, precluding a reliable measure of the \( K_{CR} \) values (data not shown).

DNA Cleavage Specificity—The reduced efficiency of the SDS-induced DNA breakage by the mutant proteins could, in principle, be due to the sequence of the particular duplex oligonucleotide substrate used in the assays. For example, WT topo70 and the mutant proteins may have different preferences for nucleotide sequence at the sites where they bind and cleave the DNA. To address this possibility, we examined the pattern of SDS-induced DNA breakage by both WT topo70 and the mutant enzymes using a 3’ end-labeled 136-bp DNA that contains multiple topoisomerase I cleavage sites, including the strong cleavage site from the rDNA of *T. thermophilus* (14, 22). The reduction in cleavage for the mutant proteins relative to WT topo70 followed the same order as described above for the magnitude in the shift in the \( K_{CR} \) (WT > H632N > H632Q > H632A ≈ H632W) (Fig. 7, −CPT). Although the cleavage pattern for the H632N mutant was very similar to that of the WT enzyme, there was insufficient cleavage with the other mutant proteins to make the comparison.

CPT is a topoisomerase I poison that enhances SDS-induced cleavage by slowing the religation phase of the reaction (36–38). By including CPT in the reactions, DNA cleavage was increased to a level that permitted a determination of the cleavage specificity of the other mutant proteins in relation to that of WT topo70. Since CPT alters the specificity of the WT enzyme somewhat (39, 40), it was important to determine the cleavage patterns of both the WT and mutant topo70 enzymes in the presence of the drug. In the presence of CPT, the cleavage patterns for the H632A and H632Q enzymes were again very similar to the WT topo70 pattern (Fig. 7, +CPT). Although only minimal cleavage was detected for H632W, the observed cleavage products also co-migrated with the WT topo70 cleavage products. These results indicate that the reduced cleavage observed for the mutant proteins was not due to an alteration in sequence specificity.

**Effect of pH on the Cleavage Rate**—Since the crystal structure of human topoisomerase I shows that His632 is proximal to the 5’-bridging oxygen of the scissile phosphate, we suggested previously that the histidine side chain might possibly serve as a general acid to donate a proton to the leaving 5’-hydroxyl as cleavage occurs (5). If His632 were to act as a general acid, deprotonation of the imidazole ring with increased pH should reduce the rate of the cleavage reaction for WT topo70, but a similar increase in pH should have no effect on cleavage by the H632Q mutant enzyme. To test this prediction, we measured the cleavage rates of both the WT and the H632Q mutant topo70 proteins at the following pH values: 6.0, 6.5, 7.2, 7.4, 7.6, 8.4, 8.9, and 9.4. As shown in Fig. 8, the activity of WT topo70 decreases slightly over the pH range from 7.6 to 9.4, but the response of the H632Q mutant enzyme was very similar. Thus, it appears unlikely that His632 acts as a general acid to donate a proton to the leaving 5’-oxygen.

**DISCUSSION**

Given the structural similarity of vaccinia topoisomerase to core subdomains I and III of human topoisomerase I (24), it is of interest to compare the properties of human topoisomerase I mutant proteins with changes at position 632 with vaccinia variants containing mutations at the corresponding His636 residue (26). Unlike with the human enzyme, changing His626 in the vaccinia enzyme to either glutamine or asparagine resulted in only a slight reduction in enzyme activity (approximately 3-fold). Similar to the H632A mutation, a H265A mutation in the vaccinia enzyme had a major effect on enzyme activity, but the magnitude of the reduction for vaccinia topoisomerase was only 100-fold compared with the almost 4 orders of magnitude observed here for the human enzyme. Although this particular histidine residue is clearly essential for the transesterification reaction catalyzed by the two enzymes, changes at this position appear to perturb the corresponding active sites somewhat differently.

In all of the available crystal structures of human topoisomerase I in noncovalent complexes with DNA (5, 18, 23), the N2-atom of His632 is positioned within hydrogen-bonding

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

**FIG. 5. Suicide cleavage assays.** WT topo70 and the mutant topo70 enzymes were incubated with the partially duplex suicide substrate (CL14:CP25) shown at the top of the figure (recognition sequence shown in bold; arrow marks cleavage site), and the reactions were stopped with SDS at a series of time points. After trypsin treatment, the amount of suicide cleavage was quantified by electrophoresis in a urea-polyacrylamide gel. The results were normalized, and the percent cleavage values were plotted for each of the enzymes.

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
<th>( k_c ) value <em>a</em></th>
<th>( K_{CR} ) <em>b</em></th>
<th>( k_c ) (calculated) <em>c</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>( 3.60 \times 10^{-4} ) s⁻¹</td>
<td>0.025 ± 0.002</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>H632Q</td>
<td>3.8 ± 0.6</td>
<td>0.004 ± 0.001</td>
<td>0.09 ± 0.06</td>
</tr>
<tr>
<td>H632N</td>
<td>2.0 ± 0.5</td>
<td>0.016 ± 0.003</td>
<td>0.013 ± 0.007</td>
</tr>
<tr>
<td>H632A</td>
<td>0.06 ± 0.01</td>
<td>NA*</td>
<td>NA</td>
</tr>
<tr>
<td>H632W</td>
<td>0.025 ± 0.004</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* All values listed are the average (±S.D.) of three determinations at 23 °C. Suicide cleavages at both the major and minor sites on CL14:CP25 were included in the calculation of \( k_c \).

*b* Calculated according to the formula \( k_c = k_d/K_{CR} \) where \( k_d \) is the suicide cleavage rate measured with the CL14:CP25 substrate.

* NA, not applicable. The slow cleavage rate precluded an accurate determination of the percent cleavage at equilibrium.
distance of the nonbridging O-2P atom of the scissile phosphate. It was hypothesized previously (5) that this interaction contributes to catalysis by stabilization of the pentavalent transition state intermediate. The 6000-fold reduction in $k_{cl}$ on changing His$_{632}$ to alanine is consistent with this hypothesis and represents strong support for an essential role of His$_{632}$ in the cleavage reaction.

Although the crystal structure of human topoisomerase I appears to rule out a role for His$_{632}$ as a general base in the activation of the nucleophilic tyrosine (5, 18), the proximity of this histidine to the 5'-oxygen of the leaving sugar (3.9 Å) suggests that in addition to stabilizing the transition state through hydrogen bonding to the nonbridging oxygen, this same residue might also act as a general acid catalyst and protonate the leaving oxygen on the sugar (5). Substituting a glutamine for His$_{632}$ provides one test of this hypothesis. When glutamine is modeled in place of His$_{632}$, all of the allowable rotamers are within hydrogen-bonding distance of the scissile phosphate nonbridging O-2P atom (3.0 Å compared with 2.8 Å for histidine), and therefore some transition state stabilization by the H632Q mutant enzyme would still be expected. However, unlike histidine, a glutamine side chain is unable to act as a general acid. The $\sim$100-fold reduction in the rates of relaxation and suicide cleavage for the H632Q mutant enzyme could be explained by the loss of general acid catalysis while retaining some interaction with the nonbridging phosphate oxygen. A second test of the possible general acid character of His$_{632}$ is to compare the pH dependence of catalysis by WT topo70 and the H632Q mutant topo70. For His$_{632}$ to donate a proton to the leaving 5'-oxygen, the amino acid side chain must be protonated. Since the normal pK$_a$ of histidine side chains in proteins ranges from 5 to 8 (41), at pH values $>8$ it would be predicted that the rate of cleavage by the WT enzyme should be substantially reduced and that the rate for the H632Q mutant would not exhibit a similar pH dependence. However, the pH profile of the activity of the WT enzyme parallels that of the
H632Q mutant enzyme with only minimal loss of activity at the higher pH values. An unlikely explanation that we cannot completely rule out is that the minimal effect of increasing pH on the WT reaction rate results from an activation of the tyrosine nucleophile by production of a phenolate anion that is just offset by loss of the general acid character of His\(^{632}\) by titration of the histidine. Overall, it seems most likely that the active site His\(^{632}\) is not acting as a general acid in the trans-esterification cleavage reaction and that its major role is to stabilize the pentavalent transition state through an interaction with the nonbridging oxygen of the scissile phosphate. Based on the pH profile of the enzyme (26) and a variety of kinetic analyses (42, 43), a similar conclusion has been reached for the vaccinia topoisomerase.

In a recent report, it was suggested that Lys\(^{167}\) in vaccinia topoisomerase acts as a general acid to protonate the leaving 5′-oxygen during the cleavage reaction (44). This suggestion is plausible given the relative pK\(_a\) values for the two groups, but in the absence of a crystal structure of the vaccinia enzyme with bound DNA, the exact role of this amino acid in catalysis by the vaccinia enzyme remains uncertain. The corresponding amino acid in human topoisomerase I (Lys\(^{532}\)) is hydrogen-bonded to the nonbridging O-1P atom of the scissile phosphate as well as to the O-2 atom of the pyrimidine base on the −1 nucleotide (23). Lys\(^{532}\) in the human enzyme is −4 Å away from the leaving 5′-oxygen, a distance that is consistent with a role as a general acid during the cleavage reaction. Further experimentation is required to define fully the role of Lys\(^{532}\) in the catalytic mechanism of human topoisomerase I.

As mentioned above, modeling studies indicate that glutamine and asparagine mutations at this position were not only nonfunctional as a hydrogen bond donor but also disrupts the overall architecture of the active site such that virtually all activity is lost. Consistent with this suggestion is the finding that when a tryptophan residue is modeled into the transition state stabilization may be slightly different in the two cases (46). If His\(^{632}\) is identical hydrogen-bonded to the O-2P atom of the scissile phosphate in the transition state for both the cleavage and religation reactions, then it would be expected that the glutamine and asparagine mutations at this position would have similar effects on the rates of the two reactions. Within the observed experimental error, the results presented in this study are compatible with this possibility (Table I). However, the observation that His\(^{632}\) is ordered in the crystal structure containing noncovalently bound DNA, but is disordered and not visible in the covalent complex (18), suggests that the detailed architecture of the active site may be different in the two cases. An understanding of the events that occur on the pathway of religation, including conformational changes in the enzyme, will be required to decide between these possibilities.

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