Histidine 265 Is Important for Covalent Catalysis by Vaccinia Topoisomerase and Is Conserved in All Eukaryotic Type I Enzymes*

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Vaccinia topoisomerase catalyzes DNA cleavage and rejoicing via transesterification to pentapyrimidine recognition site 5′-(C/T)CCTT in duplex DNA. The proposed reaction mechanism involves general-base catalysis of the attack by active site nucleophile Tyr-274 on the scissile phosphodiester and general-acid catalysis of the expulsion of the 5′-deoxyribose oxygen on the leaving DNA strand. The pKₐ values suggest histidine and cysteine side chains as candidates for the roles of proton acceptor and donor, respectively. To test this, we replaced each of the eight histidines and two cysteines of the vaccinia topoisomerase with alanine. Single mutants C100A and C211A and a double mutant C100A-C211A were fully active in DNA relaxation, indicating that a cysteine is not the general acid. Only one histidine mutation, H265A, affected enzyme activity. The rates of DNA relaxation, single-turnover strand cleavage, and single-turnover religation by H265A were 2 orders of magnitude lower than the wild-type rates. Yet the H265A mutation did not alter the dependence of the cleavage rate on pH, indicating that His-265 is not the general base. Replacing His-265 with glutamine or asparagine slowed DNA relaxation and single-turnover cleavage to about one-third of the wild-type rate. All three mutations, H265A, H265N, and H265Q, skewed the cleavage-religation equilibrium in favor of the covalently bound state. His-265 is strictly conserved in every member of the eukaryotic type I topoisomerase family.

The eukaryotic type I DNA topoisomerase family includes the nuclear type I enzymes and the topoisomerases encoded by vaccinia and other poxviruses. These proteins relax supercoiled DNA via a common reaction mechanism, which entails noncovalent binding of the topoisomerase to duplex DNA, cleavage of one DNA strand with concomitant formation of a covalent DNA-(3′-phosphotyrosyl)-protein intermediate, strand passage, and strand religation (1). A shared structural basis for transesterification and strand passage is inferred from the considerable amino acid sequence conservation found by alignment of the cellular and virus-encoded enzymes (1, 2). Catalytically important residues have been identified via mutational analysis of the 314-amino acid vaccinia virus topoisomerase. Three strategies have been used: (i) random mutagenesis followed by in vivo genetic selection of mutations that adversely affect enzyme activity (3, 4); (ii) site-directed mutagenesis of specific regions of the enzyme (5–7); and (iii) targeted mutagenesis of a specific class of amino acid residues irrespective of their location within the protein. The latter approach was used to identify Tyr-274 as the active site of the vaccinia enzyme, i.e. through systematic replacement of tyrosines by phenylalanines (8).

Physical mapping of the active site of yeast TOP1 to Tyr-727 (9, 10), supported by mutational analysis of the yeast, human, and vaccinia enzymes (8–12), localizes the active site tyrosines within a common sequence element, Ser-Lys-X-Tyr, situated near the carbonyl termini of all family members (2). Additional residues that we and others have identified as essential or important for covalent catalysis by the vaccinia topoisomerase are conserved in the cellular counterparts (3–7, 13). Indeed, the effects of mutations at the corresponding positions in cellular type I topoisomerases are generally concordant with the findings for the vaccinia enzyme (12, 14, 15). This suggests a common structural basis for DNA strand cleavage by the vaccinia and cellular topoisomerases.

A distinctive feature of the vaccinia topoisomerase is its specificity for cleaving duplex DNA at pentapyrimidine recognition site 5′-(C/T)CCTT (16–18). Using simple model substrates containing a single CCCTT cleavage site, Stivers et al. (19) have determined the rate constants for the cleavage and religation reactions at 20 °C and defined the rate-limiting steps under single-turnover and steady-state conditions. Analysis of the pH dependence of the rate constant for cleavage (kcl) and the internal equilibrium constant (Kᵢ) indicated the presence of two titratable groups on the enzyme (20). A reaction mechanism was proposed involving general-base catalysis of the attack by Tyr-274 on the scissile phosphodiester and general-acid catalysis of the expulsion of the 5′-deoxyribose oxygen (20). The pKₐ values point toward unperturbed histidine and cysteine side chains as candidates for the roles of proton acceptor and donor, respectively.

In the present study, we test the importance of histidines and cysteines in topoisomerase reaction chemistry by replacing each of the eight histidines and two cysteines of the vaccinia topoisomerase with alanine. All Ala-substitution mutations except one had no discernible effect on topoisomerase activity in vitro. Alanine substitution for His-265 slowed the overall rate of DNA relaxation by reducing the rates of the strand cleavage and the strand religation steps.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Mutations were introduced into the vaccinia virus topoisomerase gene by using the two-stage polymerase chain reaction-based overlap extension method (21). Plasmid pA9topo (22) was used as the template for the first-stage polymerase chain reaction. Gene fragments with overlapping ends obtained from the first-stage reactions were paired and used as template in the second-stage amplification. Products containing the entire topoisomerase gene were cloned into the T7-based expression vector pET11b (Novagen) as described (6, 7). All mutations were confirmed by dideoxy sequencing.

Topoisomerase Expression and Purification—pET11-based plasmids were transformed into Escherichia coli BL21. Topoisomerase expression was induced by infection with bacteriophage λCE6 as described (22), except that the cultures were adjusted to 1 mM isopropyl-1-thio-β-D-galactopyranoside immediately before inoculation with phage.

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Wild-type and mutant topoisomerases were purified from soluble bacterial extracts by phosphocellulose column chromatography (22). The protein concentrations of the phosphocellulose preparations were determined by using the dye-binding method (Bio-Rad) with bovine serum albumin as the standard.

**DNA Relaxation Assay—** Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.3 μg of PUC19 plasmid DNA, either 2.5 mM EDTA or 5 mM MgCl₂, and wild-type or mutant topoisomerases (12, 4, 1.3, 0.44, 0.15, 0.05, or 0.016 ng of the phosphocellulose enzyme preparations) were incubated at 37 °C for 15 min. The reactions were quenched by the addition of a solution containing SDS (0.3% final concentration), glycerol, xylene cyanol, and bromphenol blue. Samples were analyzed by electrophoresis through a 1.2% agarose gel in TBE buffer (90 mM Tris borate and 2.5 mM EDTA). The gels were stained in 0.5 μg/ml ethidium bromide solution, destained in water, and photographed under short-wave UV illumination.

**Suicide Cleavage Assay—** An 18-mer CCCTT-containing DNA oligonucleotide was 5’ end-labeled by enzymatic phosphorylation in the presence of [γ-³²P]ATP and T4 polynucleotide kinase and then gel-purified to a 30-mer strand (present at 4-fold molar excess). Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 18-mer/30-mer DNA, and topoisomerase were incubated at 37 °C. Covalent complexes were trapped by the addition of SDS to 1%. The denatured samples were electrophoresed through a 10% polyacrylamide gel containing 7M urea electrophoresed through a 17% polyacrylamide gel containing 7M urea. The cleavage product, a 32P-labeled 30-mer bound to a short peptide, was well resolved from the input 60-mer substrate (18). The extent of strand cleavage was quantitated by scanning the dried gel using a FUJIX BAS1000 Bio-Imaging analyzer.

**pH Dependence of Suicide Cleavage—** Single-turnover cleavage assays were performed at 22 °C with 50 mM of each of the following reaction buffers: sodium acetate, pH 4.6; sodium 2-(N-morpholino)ethanesulfonic acid, pH 5.6 and 6.5; Tris-HCl, pH 7.5 and 8.5, and sodium 3-(cyclohexylamino)-1-propanesulfonic acid, pH 9.5. The wild-type and H265A topoisomerases were preincubated in a 50 mM solution of reaction buffer for 5 min. The cleavage reactions were initiated by mixing the enzyme solution with an equal volume of 50 mM reaction buffer containing the DNA substrate. (Final concentrations were 50 mM buffer, 1.9 μg/ml topoisomerase, and 15 μg DNA.) To determine the rate of relaxation by the H265A mutant, aliquots (20 μl) were withdrawn at 15 and 30 s; 1, 2, 5, 10, 20, and 30 min; and 1, 2, 4, 6, 8, and 12 h. (An additional 24 h time point was taken for rate determination at pH 4.6.) The samples were quenched immediately by adding SDS. The protein-DNA adducts were resolved by SDS-polyacrylamide gel electrophoresis and quantitated by scanning the gels with a Bio-Imaging analyzer. A plot of the percentage of input DNA cleaved versus time established end-point values for cleavage. The data were normalized to the end-point values, and kobs was determined by fitting the data to the equation:

\[
100 - \frac{C_{\text{final}}}{C_{\text{initial}}} = 100e^{-k_{\text{obs}}t}
\]

Aliquots were taken from wild-type topoisomerase cleavage reaction mixtures at 10, 20, and 30 s and 1, 2, 5, 10, 20, and 60 min. To better determine the initial rates of wild-type cleavage, additional sets of reaction mixtures were quenched at a single time point (5 s). The 5-s reactions were performed in triplicate at each pH; the average 5-s value was used to calculate the cleavage rate constant.

Control experiments were performed to test whether the H265A protein was inactivated during a 24-h incubation at 22 °C at pH 4.6, 5.6, 6.5, 7.5, 8.5, or 9.5. After this incubation, the protein was adjusted to pH 7.5 and assayed for suicide cleavage during a 5-min incubation at pH 7.5. We found that the H265A protein suffered no loss of activity during these incubations.

**Equilibrium Cleavage Assay—** A 60-mer oligonucleotide containing a centrally placed CCCTT element was 5’ end-labeled and then gel-purified and annealed to an unlabeled complementary 60-mer strand. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 0.3 pmol of 60-mer DNA duplex, and topoisomerase were incubated for 10 min at 37 °C. Covalent complexes were trapped by the addition of SDS to 1%. The samples were digested for 30 min at 45 °C with 10 μg of DNAase K. The volume was adjusted to 50 μl, and the digests were then extracted with an equal volume of phenol/chloroform. DNA was recovered from the aqueous phase by ethanol precipitation. The pelleted material was resuspended in formamide, and the samples were electrophoresed through a 17% polyacrylamide gel containing 7 M urea in TBE. The cleavage product, a ³²P-labeled 30-mer bound to a short peptide, was well resolved from the input 60-mer substrate (18). The extent of strand cleavage was quantitated by scanning the wet gel using a Bio-Imaging analyzer.

**RESULTS**

Single alanine substitutions were introduced at each of the eight histidines and two cysteines of the 314-amino acid vaccinia topoisomerase. A double mutant in which both cysteines were replaced by alanine was also included in the analysis. The wild-type and mutated proteins were expressed in E. coli using a T7 RNA polymerase-based expression system (22). The recombinant proteins were purified from bacterial extracts by phosphocellulose column chromatography. The polypeptide compositions of the enzyme preparations were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). In every case, the 33-kDa topoisomerase polypeptide constituted the major species, and the extents of purification were essentially equivalent.

To assess the impact of these mutations, all proteins were tested for their ability to relax supercoiled plasmid DNA in vitro. Screening assays were performed in the absence of magnesium. (The rate-limiting step under these conditions is the dissociation of topoisomerase from the relaxed plasmid product.) Activity was quantitated by end-point dilution, beginning with 12 ng of the phosphocellulose topoisomerase preparation and decreasing by serial 3-fold decrements to 16 pg. We observed that the specific activity of every mutant protein except one (H265A) was equivalent to that of the wild-type topoisomerase (data not shown).

The DNA relaxation assays were also performed in the presence of 5 mM magnesium. Magnesium stimulates the activity of the wild-type enzyme—9-fold under conditions of DNA excess by enhancing the product off-rate without affecting the rate of DNA cleavage (19, 20). The specific activities of all the mutant proteins (except H265A) were enhanced —9-fold by 5 mM magnesium and were again equivalent to that of the wild-type enzyme (data not shown).

Hence, we conclude that Cys-100 and Cys-211 are dispensable for topoisomerase activity and that seven of the histidines (His-33, His-59, His-76, His-152, His-172, His-177, and His-307) are nonessential. The experiments that follow focus on the catalytic contributions of His-265.

**H265A Is Defective in Relaxing Supercoiled Plasmid DNA—**
H265A Affects the Rate of DNA Cleavage—Suicide cleavage was assayed as described under "Experimental Procedures." The structures of the 5'-32P-labeled suicide substrate and the covalent topoisomerase-DNA adduct are depicted at the top of the figure. A, reaction mixtures containing 0.3 pmol of DNA substrate and the indicated amounts (in nanograms) of wild-type (WT) or H265A protein were incubated for 5 min at 37 °C. The extent of covalent adduct formation is plotted as a function of protein. B, reaction mixtures containing (per 20 μl) 0.3 pmol of DNA substrate and 38 ng of wild-type (WT) or H265A protein were incubated at 37 °C. The cleavage reactions were initiated by the addition of topoisomerase to prewarmed reaction mixtures. Aliquots (20 μl) were withdrawn at 10, 20, and 30 s and 1, 2, 5, 10, 20, and 60 min and quenched immediately by adding SDS. Covalent complex formation is plotted as a function of time.

Suicide cleavage by the wild-type topoisomerase was nearly complete within 10 s at 37 °C (Fig. 3B). In contrast, H265A cleaved the DNA quite slowly. Covalent adduct accumulated steadily over 20 min; 84% of the input substrate was cleaved after 1 h (Fig. 3B). The H265A data fit well to a single exponential with an apparent cleavage rate constant (kobs) of 0.002 s−1. The extent of cleavage by wild-type enzyme at 5 s was 76% of the end-point value (+4%; average of five experiments). We used this datum to estimate a wild-type rate constant of 0.28 s−1. Thus, we observed that the H265A mutation slowed the rate of cleavage by 2 orders of magnitude. Note that kobs for wild-type topoisomerase at 37 °C was higher than the value of 0.07 s−1 determined at 20 °C with a different DNA substrate (19).

Is His-265 the General Base?—It was hypothesized previously that a histidine might function as a general base during transesterification (20). According to this model, the imidazole ring nitrogen would accept a proton from the hydroxyl of the active site tyrosine (Tyr-274), thereby facilitating nucleophilic attack by the phenolic oxygen on the scissile phosphate. If His-265 plays such a role, we would expect the H265A mutant to display an altered pH-rate profile in single-turnover cleavage. We therefore measured the rate of suicide cleavage by H265A as a function of pH in the range of pH 4.6–9.5. A plot of log kobs versus pH is shown in Fig. 4. The shape of the H265A pH-rate profile was similar to that of wild-type topoisomerase. This argued that His-265 is not the general base in topoisomerase-mediated strand cleavage.

Substitution of His-265 with Asn and Gln—His-265 was replaced with glutamine and asparagine, which are nearly isoelectric with histidine (30) but cannot be protonated like histidine. The H265N and H265Q proteins were expressed in bacteria and purified by phosphocellulose chromatography. The polypeptide compositions of these enzyme preparations were similar to that of the wild type depicted in Fig. 1 (data not shown). The rates of relaxation of supercoiled plasmid DNA by H265N and H265Q in the absence of a divalent cation were about one-half to one-fourth of the wild-type rate (Fig. 5).
Relaxation by H265N and H265Q was stimulated 2-fold by 5 mM EDTA (or 5 mM MgCl₂) and the DNA. This transesterification step can be studied covalently bound at saturation. The mild effects of the H265N and H265Q mutations on the rate of single-turnover DNA cleavage contrasted with the severe rate decrement observed for the H265A mutant.

Effect of H265 Mutations on DNA Religation—Religation of the cleaved strand occurs by attack of a 5'-OH-terminated polynucleotide on the 3' phosphodiester bond between Tyr-274 and the DNA. This transesterification step can be studied independent of strand cleavage by assaying the ability of a preformed topoisomerase-DNA complex to religate the covalently held 5'-32P-labeled strand to a heterologous acceptor strand. The wild-type, H265N, H265Q, and H265A proteins were incubated with the suicide cleavage substrate for 60 min to attain near-equivalent levels of the covalent intermediate. We then added a 100-fold molar excess of an 18-mer acceptor strand complementary to the 5' tail of the covalent donor complex, while simultaneously increasing the ionic strength to 0.3 M NaCl. (Addition of NaCl during the religation phase promotes dissociation of the topoisomerase after strand closure and prevents recleavage of the strand transfer product.) Religation to the 18-mer yielded the 32P-labeled 30-mer depicted in Fig. 7. The strand transfer product was resolved from the input 32P-labeled 18-mer strand by denaturing gel electrophoresis.

The wild-type enzyme transferred 96% of the input CCCTT-containing strand to the exogenous acceptor (Fig. 7). The extent of religation at the earliest time point analyzed (10 s) was 95% of the end-point value. Similarly, H265N and H265Q transferred >90% of the input DNA to the acceptor, with ~80% of the end-point value attained in 10 s. Thus, the Asn and Gln substitutions caused a relatively mild slowing of the strand transfer reaction. In contrast, strand transfer by H265A was much slower. The religated 30-mer accumulated steadily over 10 min; 74% of the input substrate was religated after 20 min. The observed religation rate constant (k_rel) was 0.004 s⁻¹. Thus, the H265A mutation slowed the rate of religation by at least 2 orders of magnitude relative to the wild-type religation rate.

H265 Mutations Alter the Cleavage-Religation Equilibrium in Favor of Covalent Binding—We used a 60-bp DNA duplex containing a centrally placed cleavage site with 30 bp upstream and 30 bp downstream of the scissile bond to study topoisomerase cleavage under true equilibrium conditions. Cleavage of the 60-mer duplex by the wild-type topoisomerase was linear up to 20 ng of protein and plateaued at 38–152 ng (Fig. 8). At saturation, 17% of the substrate was cleaved. The cleavage equilibrium constant (K₉ = covalent complex/noncovalent complex) was 0.2, which was slightly higher than the K₉ of 0.13 deter-
FIG. 7. Effect of the H265A, H265N, and H265Q mutations on single-turnover DNA religation. Cleavage reaction mixtures containing (per 20 μl) 0.3 pmol of labeled 60-bp DNA substrate and 38 ng of wild-type, H265A, H265N, or H265Q proteins were incubated for 60 min at 37°C. At this point (time 0), an aliquot was removed and quenched immediately by adding SDS and 0.4% SDS, 95% formamide, and 20 mM EDTA. Religation was initiated by the simultaneous addition of NaCl to 0.3 M and an 18-mer acceptor strand to a concentration of 30 pmol/20 μl (i.e., a 100-fold molar excess over the input DNA substrate). Religation to the heterologous acceptor will yield the 5′→3′ ligation product shown at the right. Aliquots (20 μl) were withdrawn at 10, 20, and 30 s and 1, 2, 5, 10, and 20 min after the addition of NaCl. The extent of religation (expressed as the percentage of the input labeled 30-mer strand recovered as 30-mer) is plotted as a function of reaction time.

FIG. 8. Effect of H265A, H265N, and H265Q mutations on equilibrium DNA cleavage. Covalent complex formation on a 60-bp duplex substrate was assayed as described under "Experimental Procedures." The structure of the substrate is shown with the site of cleavage indicated by a vertical arrow. Reaction mixtures (20 μl) containing 0.3 pmol of labeled 60-bp DNA and the indicated amounts (in nanograms) of wild-type, H265A, H265N, or H265Q proteins were incubated for 5 min at 37°C. The extent of covalent complex formation is plotted as a function of input protein.

FIG. 9. Approach to equilibrium and single-turnover religation on the 60-mer DNA. A, approach to equilibrium. Reaction mixtures containing (per 20 μl) 0.3 pmol of labeled 60-bp DNA and 76 ng of H265A, H265N, or H265Q protein. The reactions were initiated by adding topoisomerase to prewarmed 37°C reaction mixtures. Aliquots (20 μl) were withdrawn at 10, 20, and 30 s and 1, 2, 5, and 10 min and quenched immediately by adding SDS. Covalent complex formation is plotted as a function of time. B, single-turnover religation. Cleavage reaction mixtures containing (per 20 μl) 0.3 pmol of labeled 60-mer duplex DNA and 76 ng of H265A, H265N, or H265Q protein were incubated for 10 min at 37°C. At this time (time 0), an aliquot (20 μl) was removed and quenched with SDS. The reaction mixtures were then adjusted to 0.3 M NaCl, and aliquots (20 μl) were incubated at 10, 20, and 30 s and 1, 2, 5, and 10 min. The decrease in the abundance of the covalent complex (expressed as a percentage of input DNA) is plotted as a function of time.
The hydrolysis of the phosphodiester bond is catalyzed by a conserved histidine residue, His-265, in the vaccinia topoisomerase. This residue is situated only 9 amino acids away from the active site of the enzyme, suggesting a critical role in the catalytic process.

His-265 is essential for topoisomerase activity, as its absence leads to a decrease in the rate of DNA relaxation compared to the wild-type protein. The crystal structure of topoisomerase bound to DNA has provided insights into the mechanism of DNA cleavage and religation. The conserved histidine plays an essential role in the chemistry of the reaction, functioning as a general base during the reaction.

The implications of this finding are significant, as they suggest that His-265 is a key determinant of the catalytic efficiency of topoisomerases. Understanding the role of this residue may provide new insights into the design of antiviral drugs targeting topoisomerases.

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