

# Regulation of Catalysis by the Smallpox Virus Topoisomerase\*<sup>§</sup>

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The poxvirus type IB topoisomerases catalyze relaxation of supercoiled DNA by cleaving and rejoining DNA strands via a pathway involving a covalent phosphotyrosine intermediate. Recently we determined structures of the smallpox virus topoisomerase bound to DNA in covalent and non-covalent DNA complexes using x-ray crystallography. Here we analyzed the effects of twenty-two amino acid substitutions on the topoisomerase activity *in vitro* in assays of DNA relaxation, single cycle cleavage, and equilibrium cleavage-religation. Alanine substitutions at 14 positions impaired topoisomerase function, marking a channel of functionally important contacts along the protein-DNA interface. Unexpectedly, alanine substitutions at two positions (D168A and E124A) accelerated the forward rate of cleavage. These findings and further analysis indicate that Asp<sup>168</sup> is a key regulator of the active site that maintains an optimal balance among the DNA cleavage, religation, and product release steps. Finally, we report that high level expression of the D168A topoisomerase in *Escherichia coli*, but not other alanine-substituted enzymes, prevented cell growth. These findings help elucidate the amino acid side chains involved in DNA binding and catalysis and provide guidance for designing topoisomerase poisons for use as smallpox antivirals.

All poxviruses encode topoisomerases of the type IB family (1, 2). These enzymes release DNA supercoils via a mechanism involving a 3'-phosphotyrosine covalent protein-DNA intermediate (Fig. 1A). In this reaction, the topoisomerase first binds to DNA, then catalyzes nucleophilic attack of an enzyme tyrosine residue on one DNA strand, forming a 3'-phosphotyrosine linkage. This resulting DNA strand break allows rotation of the continuous DNA strand around the nick, thereby releasing supercoils (3, 4). The reaction cycle is completed by religation, in which the hydroxyl group of the free DNA 5'-end at the nick attacks the phosphotyrosine linkage, rejoining the DNA, followed by product release (5, 6).

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1.

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Topoisomerases are the targets of several classes of drugs that are important in anti-cancer and anti-microbial chemotherapy. These "topoisomerase poisons" act by trapping the covalent protein-DNA complex, thereby stabilizing toxic DNA breaks. Accumulation of DNA breaks then poisons cancer cells or infecting microbes. The poxvirus topoisomerases are thus attractive drug targets for poxvirus-specific topoisomerase poisons (7–11).

The poxvirus topoisomerases are unique for acting only at a conserved sequence, which consists of a core 5'-(T/C)CCTT-3' motif and optimal flanking sequences (12–14) (Fig. 1B). Previous studies have shown that the sequence-specific recognition is involved not only in initial DNA binding but in a postbinding conformational step that promotes cleavage (4, 6, 15). Very extensive mutagenesis of the vaccinia topoisomerase has identified residues involved in catalysis and additional residues important for activation of cleavage after DNA binding (4, 6, 15–21).

The elucidation by x-ray crystallography of two structures of smallpox virus topoisomerase bound to its DNA recognition site has allowed enzyme activation to be understood in structural terms (22). The poxvirus topoisomerases are two-domain proteins (22–25) that bind to the 5'-(T/C)CCTT-3' site as monomers by forming C-shaped clamps around the DNA (22, 23, 26). Comparison of the vaccinia amino and carboxyl domain structures determined in the absence of DNA (23, 25) to the smallpox-topoisomerase/DNA complex (22) allowed the conformational changes that mediate activation to be visualized. The N-terminal domain binds to DNA essentially as a rigid body, but the C-terminal catalytic domain undergoes a considerable structural reorganization upon binding (22). Multiple protein side chains along the DNA interface make contacts that are positioned to stabilize the active conformation. Particularly notable is the formation of  $\alpha$ -helix 5. This segment of the protein is disordered in the absence of DNA (23), but upon binding to the favored 5'-(T/C)CCTT-3' site, forms an  $\alpha$ -helix that inserts into the DNA major groove (22). Amino acid side chains projecting from helix 5 make specific contacts to base pairs +3 to +6 (for numbering of the DNA base pairs in the recognition site see Fig. 1B). Formation of helix 5 allows nearby segments of the amino acid chain to bind DNA and deliver one of the catalytic residues (Arg<sup>130</sup>) to the vicinity of the scissile phosphate, thereby activating covalent chemistry. Other notable topoisomerase-DNA interactions involve: 1)  $\beta$ -strand 5, which inserts into the DNA major groove and makes sequence-specific interactions to base-pairs +2 to +4, 2) Arg<sup>206</sup>, which makes an upstream contact to a base pair edge at +9, and 3) the

**TABLE 1**  
Summary of the activities of topoisomerase mutants

Name of mutant	Relaxation (time to 50% conversion)	Cleavage ( $k_{cl}$ ) (suicide substrate)	Proportion of covalent complex formation ( $K_{cl}$ ) (equilibrium substrate)	Religation rate ( $k_{rel}$ ) (calculated)
	min	$1/s (\times 10^{-2})$		$1/s (\times 10^{-2})$
Wild type	5 ± 1	17 ± 6		
C100S	6 ± 0	17 ± 1		
C211S	9 ± 1	1.8 ± 0.1		
C100S C211S	10.4 ± 2.7 <sup>a</sup>	1.9 ± 1.0	0.13 ± 0.01	14.61
CSCS H39A	12 ± 0	0.023 ± 0.018	0.08 ± 0.01	0.29
CSCS Q69A	37 ± 5 <sup>a</sup>	0.029 ± 0.023	0.02 ± 0.00	1.45
CSCS R80A	36 ± 4	0.087 ± 0.062	0.04 ± 0.01	2.18
CSCS E124A	11.2 ± 5 <sup>a</sup>	9.7 ± 1.60	0.16 ± 0.11	60.63
CSCS E124Q	6 ± 2 <sup>a</sup>	1.05 ± 0.07	0.06 ± 0.01	17.50
CSCS R130A	>1440 <sup>a</sup>	0.0041	Did not reach equilibrium	
CSCS R130M	>1440			
CSCS K133A	38 ± 6 <sup>a</sup>	0.113 ± 0.109	0.02 ± 0.00	5.65
CSCS K135A	21 ± 10 <sup>a</sup>	1.40 ± 0.57	0.07 ± 0.01	20.00
CSCS K167A	>1440 <sup>a</sup>	Undetectable	Did not reach equilibrium	
CSCS K167M	>1440			
CSCS D168A	641 ± 59 <sup>a</sup>	24.7 ± 5.5	0.36 ± 0.05	68.61
CSCS R206A	8 ± 0 <sup>a</sup>	0.044 ± 0.012	0.05 ± 0.02	0.88
CSCS Y209A	9.6 ± 3.5 <sup>a</sup>	0.047 ± 0.026	0.02 ± 0.00	2.35
CSCS R223A	>1440 <sup>a</sup>	Undetectable	Did not reach equilibrium	
CSCS R223M	>1440			
CSCS H265A	325 ± 25 <sup>a</sup>	0.00086 ± 0.00041	0.35	0.0025
CSCS T266A	9 ± 1	0.079 ± 0.064	0.08 ± 0.00	0.98
CSCS Y274F	>1440 <sup>a</sup>	Undetectable	Undetectable	

<sup>a</sup> Indicates previously tested (Ref. 22).

active site residues (Arg<sup>130</sup>, Lys<sup>167</sup>, Arg<sup>223</sup>, His<sup>265</sup>, Tyr<sup>274</sup>), which make a network of contacts involving residue +1 and the scissile phosphate (22).

The topoisomerase/DNA complex structures, together with extensive previous work, have led to a detailed picture of the catalytic chemistry. The reaction is catalyzed by a combination of acid-base catalysis, in which the attacking nucleophile is deprotonated and the leaving group protonated, and electrostatic stabilization, in which the developing negative charge at the scissile phosphate is stabilized by basic side chains at the active site (Refs. 4, 6, 15, 27 and references therein). A study of the topoisomerase enzyme of *Leishmania* bound to DNA and containing vanadate, a transition state analog, at the position of the scissile phosphate has yielded a view of the probable transition state (28), confirming and extending the models developed in the poxvirus and human topoisomerase systems.

Here we present a study of amino acid side chains of the smallpox topoisomerase that were suggested by the structural analysis to have a functional role. We focused particularly on amino-acids at the protein-DNA interface that were not thoroughly studied in previous work in the vaccinia system. The effects of amino acid substitutions were analyzed *in vitro* in assays for full DNA relaxation or the individual steps in the topoisomerase reaction cycle. This work revealed a network of new contacts important for enzyme function, and unexpectedly also disclosed amino acid substitutions that increased the apparent rate of cleavage of suicide substrates *in vitro*.

## EXPERIMENTAL PROCEDURES

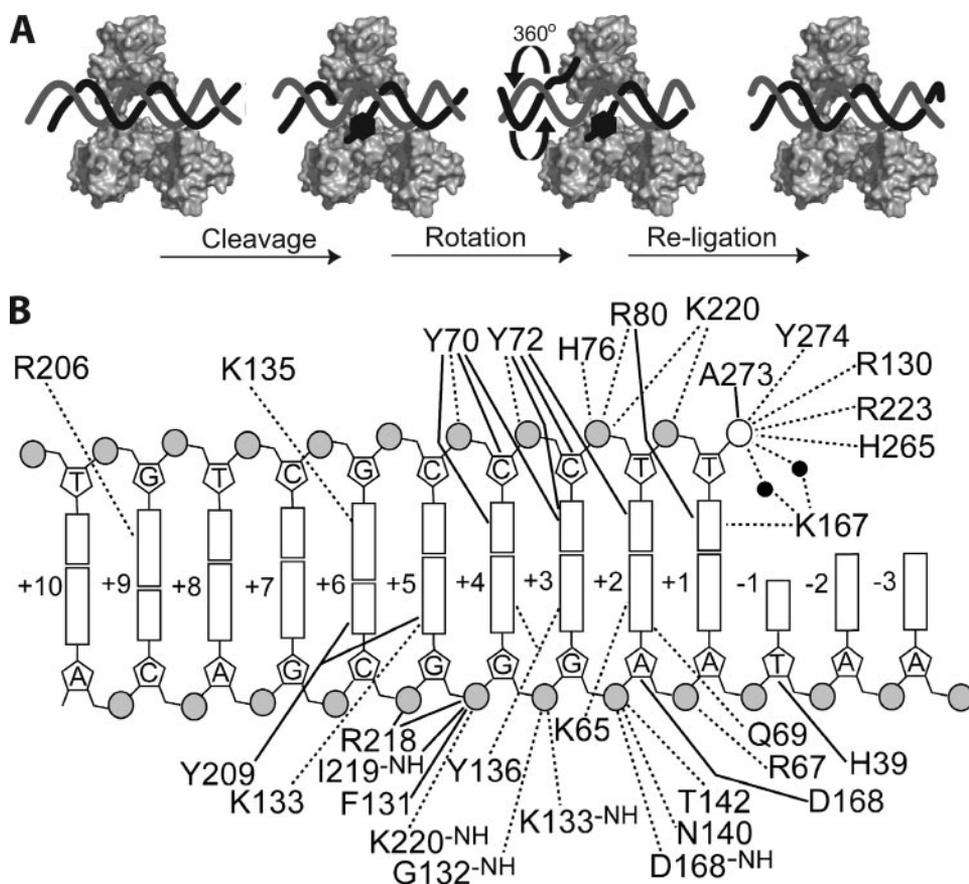
**Site-directed Mutagenesis**—Mutations were introduced into the variola double cysteine mutant topoisomerase gene by using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). Plasmid pET29a double cysteine mutant VAR-TOPO was the template for the mutagenesis reaction. All mutations were confirmed by dideoxy DNA sequencing method.

**Topoisomerase Expression and Purification**—pET29-based plasmids containing mutant topoisomerase genes were transformed into *Escherichia coli* BL21(DE3) Codon-Plus RIL (Stratagene, La Jolla, CA) and were induced by adding IPTG. Double cysteine and mutant topoisomerases were purified from soluble bacterial lysates using SP-Sepharose column chromatography. The protein concentrations of the SP-Sepharose preparations were determined by using the dye binding method (Bio-Rad) with bovine serum albumin as the standard.

**DNA Relaxation Assay**—Reaction mixtures (10  $\mu$ l) containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1  $\mu$ g of pUC19 plasmid DNA and 4.6 ng of double cysteine or mutant topoisomerases (4-fold molar excess of plasmid molecules over topoisomerase molecules) were incubated at 25 °C for appropriate time. The reactions were stopped by the addition of a solution containing SDS (2% final concentration), glycerol, and bromphenol blue. Samples were analyzed by electrophoresis through a 1.0% horizontal agarose gel in TAE buffer (40 mM Tris acetate and 1 mM EDTA). The gels were stained in 0.5  $\mu$ g/ml ethidium bromide solution and photographed. The density of bands was measured by scanning the photograph using a STORM Phospho-Imaging Analyzer. All assays in this study were repeated at least twice to estimate error.

**Forward Cleavage Assays**—A 16-mer CCCTT-containing DNA oligonucleotide was 5' end-labeled by enzymatic phosphorylation in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase and annealed to a complementary 18-mer bottom strand. Cleavage reaction mixtures containing (per 20  $\mu$ l) 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 8 pmol of 16-mer/18-mer DNA, and topoisomerase were incubated at 25 °C. Covalent complexes were denatured by addition of SDS to 2%. The samples were electrophoresed through a 12% polyacrylamide gel

## Catalysis by the Smallpox Virus Topoisomerase



**FIGURE 1. Mechanism of catalysis by the smallpox virus topoisomerase.** *A*, reaction scheme for DNA relaxation by a type IB topoisomerase. The structure of the smallpox topoisomerase is indicated by the gray envelope. The black hexagon indicates formation of the covalent topoisomerase-DNA intermediate. *B*, map of DNA-protein interactions in the smallpox topoisomerase/DNA complex deduced from data in Ref. 22. Dashed lines are hydrogen bonds less than or equal to 3.3 Å (except for Tyr<sup>274</sup>, which is covalent). Solid lines are Van der Waals contacts less than or equal to 4.0 Å.

versus time established end point values for cleavage.  $k_{\text{obs}}$  was determined by fitting the data to the equation  $(100 - \% \text{ cleaved product}) = 100e^{-kt}$  using the GraphPad PRISM version 4.

**Equilibrium Cleavage Assays**—A 36-mer oligonucleotide containing CCCTT was 5' end-labeled and annealed to a complementary 36-mer strand. Cleavage reaction mixtures (20  $\mu\text{l}$ ) containing 20 mM Tris-HCl, pH 8.0, 8 pmol of 36-mer DNA duplex, and topoisomerase were incubated at 25 °C. Covalent complexes were denatured by the addition of SDS to 2%. The samples were electrophoresed through a 12% polyacrylamide gel containing 0.1% SDS. The cleavage product, a <sup>32</sup>P-labeled 12-mer covalently linked to topoisomerase, was well resolved from the input 36-mer substrate. The extent of strand cleavage was quantitated by scanning the gel with a STORM Phospho-Imaging Analyzer.

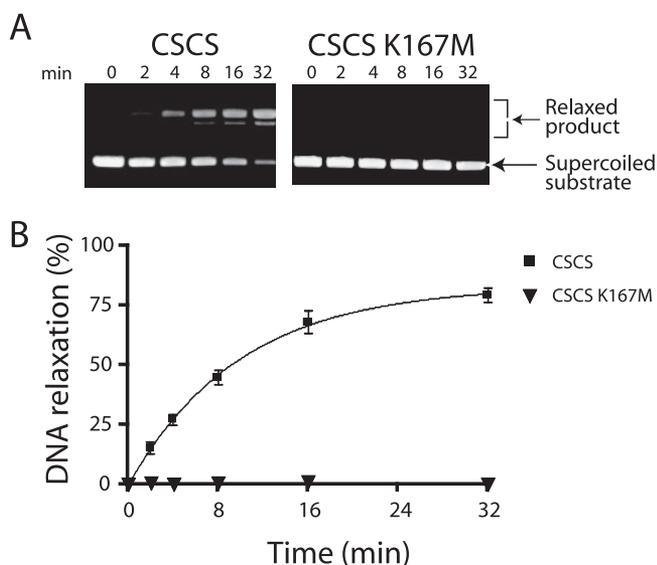
## RESULTS

**Selection of Amino Acids for Analysis**—The amino acid substitutions studied in this work are listed in Table 1. All mutants were generated in a gene encoding the small-

pox virus topoisomerase that also contained the substitutions C100S and C211S (termed "CSCS" below). These substitutions were required for formation of diffraction quality crystals of the smallpox topoisomerase with DNA (22), and so were included here to allow direct comparison with structural data. The C211S substitution diminished the activity of the enzyme slightly (Table 1), which had the advantage of slowing the reaction rates and simplifying some of the kinetic studies.

The DNA-contacting residues (Fig. 1*B*) were typically substituted with alanine to allow the effects of selective truncation of the side chain to be studied. Residue His<sup>39</sup> projects off the N-terminal domain and makes a contact to the T at position -1. Gln<sup>69</sup> projects off  $\beta$ -5 of the N-terminal domain, and makes a sequence-specific contact to the major groove at position +2. Arg<sup>80</sup> projects from  $\alpha$ -helix 3, which connects the N- and C-terminal domains, and contacts the Thy at +1. Lys<sup>133</sup> and Lys<sup>135</sup> extend from helix 5 and make major groove contacts at positions +5 and +6. Arg<sup>206</sup> makes the most upstream contact, extending to the +9 position. Tyr<sup>209</sup> contacts +6 and Thr<sup>266</sup> is positioned to contact -1 (though this part of the DNA is missing in the structures).

The active site residues (Arg<sup>130</sup>, Lys<sup>167</sup>, Arg<sup>223</sup>, His<sup>265</sup>, and Tyr<sup>274</sup>) have all been studied previously in the vaccinia topoisomerase background (4, 6, 15, 17–20). Here we substituted these residues with alanine, as in previous work. Catalytic site



**FIGURE 2. Assays of relaxation of plasmid DNAs by mutant derivatives of the smallpox virus topoisomerase.** *A*, sample relaxation time courses analyzed by gel electrophoresis and staining with ethidium bromide. The mutant enzymes studied and times are indicated above the gels. *B*, quantification of the gels in *A*.

containing 0.1% SDS. The extent of covalent adduct formation was quantitated by scanning the gel using a STORM Phospho-Imaging Analyzer. A plot of the percent of input DNA cleaved

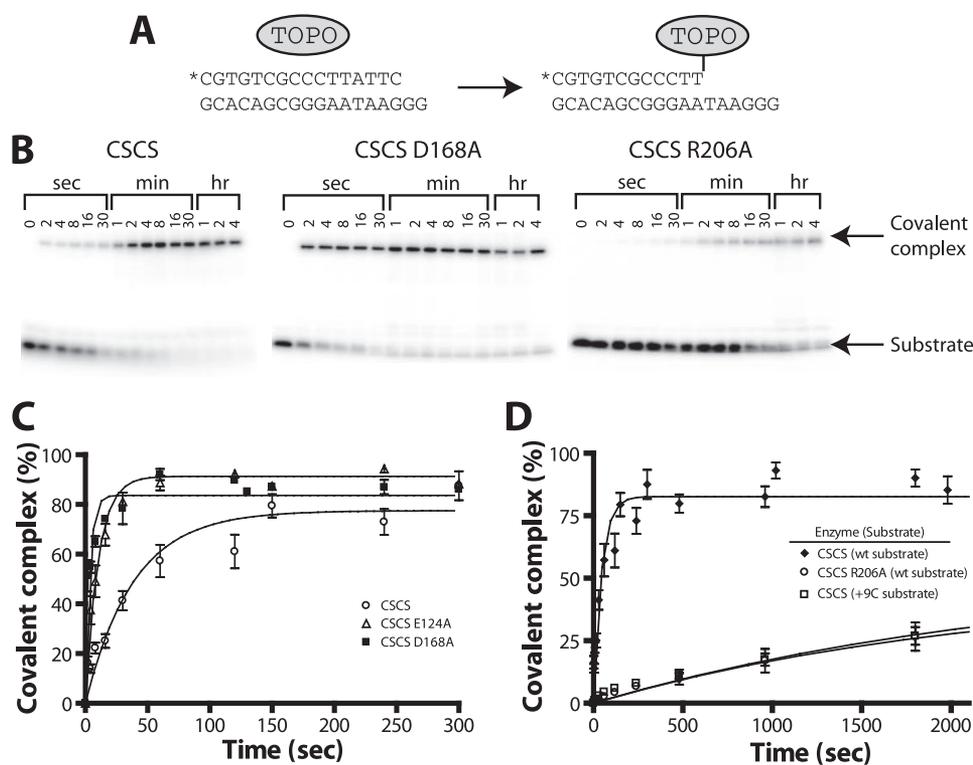


FIGURE 3. Assays of cleavage on suicide substrates. *A*, trapping covalent complexes using suicide substrates. The DNA 5'-end labeled with  $^{32}\text{P}$  is indicated by the asterisk. *B*, autoradiograms illustrating time course analysis of cleavage by the CSCS reference topoisomerase, CSCS D168A, and CSCS R206A. Titration of the CSCS topoisomerase indicated that the saturating amounts of enzyme were used in the experiment (data not shown). *C*, quantification of assays for CSCS, CSCS E124A, and CSCS D168A. *D*, time course studies of the effects of the R206A substitution in CSCS or the +9 C substitution in the DNA on cleavage rate.

TABLE 2  
Loss of discrimination at position +9 with the R206A substitution

Substrate	Rate	
	VAR-TOPO CSCS	VAR-TOPO CSCS R206A
	$1/S \times 10^{-2}$	
WT	1.93 $\pm$ 1.00 (100%)	0.044 $\pm$ 0.012 (2.3%)
+10 Substitutions		
G	0.52 $\pm$ 0.19 (26.9%)	0.026 $\pm$ 0.013 (1.4%)
+9 Substitutions		
A	0.22 $\pm$ 0.07 (11.4%)	0.047 $\pm$ 0.020 (2.4%)
T	0.17 $\pm$ 0.04 (8.8%)	0.082 $\pm$ 0.042 (4.2%)
C	0.04 $\pm$ 0.01 (2.1%)	0.046 $\pm$ 0.004 (2.4%)
+8 Substitutions		
G	1.20 $\pm$ 0.14 (62.2%)	0.055 $\pm$ 0.0042 (2.8%)
+2 Substitution		
A	0.013 $\pm$ 0.003 (0.67%)	<10 $^{-5}$

residues Arg<sup>130</sup>, Lys<sup>167</sup>, and Arg<sup>223</sup> were also substituted with methionine in order to maintain similar hydrophobic contacts along the length of the side chain, while selectively eliminating the charged functional group, a type of substitution not previously studied. The Tyr<sup>274</sup> catalytic tyrosine was substituted with Phe to remove the hydroxyl group that serves as the nucleophile in the transesterification reaction mediating initial cleavage, a type of substitution also studied previously (16, 29).

Residue Asp<sup>168</sup> was implicated as potentially important because it lies on the outer rim of the active site, positioned to interact with the 5'-hydroxyl group at the scissile phosphate, and well positioned to participate in the religation reaction. In the structures, the main chain NH of Asp<sup>168</sup> contacts the DNA

phosphate on the 3'-side of +2. The Asp<sup>168</sup> side chain is available to contact the cleaved DNA strand in the vicinity of -1 or -2, but this portion of the DNA is missing in the available structures so detailed interactions are uncertain. An initial study of a D168A topoisomerase derivative indicated that this side chain was important for DNA relaxation (22), but did not specify the step involved.

Residue Glu<sup>124</sup> is an unusual buried acidic residue. The side chain is in a generally hydrophobic environment in the protein interior, but it does have the potential to exchange protons through a series of relays to the active site. Possibly the relay system involves an imidic acid tautomer of the peptide backbone (22). Previous studies of the effects of E124A on DNA relaxation in the vaccinia (21) and variola enzymes (22) showed no strong reduction in rate.

Topoisomerase derivatives containing each of these substitutions were analyzed for their effects on DNA relaxation, the forward rate of

DNA cleavage, and the position of the equilibrium between cleavage and religation. In what follows, mutant derivatives are referred to as "CSCS" followed by specifying the substitution (e.g. CSCS D168A).

*Effects of Amino Acid Substitutions on DNA Relaxation*—DNA relaxation was analyzed using supercoiled bacterial plasmids as substrates. Following incubation with topoisomerase protein, supercoiled and relaxed topoisomers were separated by electrophoresis and quantified. In these reactions, 0.13 pmol of topoisomerase were added to 0.57 pmol of plasmid, so that relaxation of the substrate required that each protein molecule carry out several cycles of DNA relaxation, product release, and rebinding to new DNAs. Sample reactions are shown in Fig. 2*A* for the CSCS reference enzyme and CSCS K167M. Results are quantified in Fig. 2*B* and summarized in Table 1. For some of the mutants, relaxation half-times have been previously reported (denoted by the asterisks in Table 1 and Ref. 22).

Substitutions in the previously identified active site residues (R130A, K167A, R223A, H265A, and Y274F) severely impaired relaxation, paralleling previous studies in the vaccinia system (4,6,15,17–20,22). The CSCS topoisomerase derivatives containing methionine substitutions R130M, K167M, and R223M were also severely reduced in activity, implicating the terminal basic charge of the side chain as important for catalysis, and arguing against the idea that creation of a cavity in the active site by the alanine substitution was the main deficit. CSCS D168A was also severely impaired. The CSCS E124A and CSCS E124Q

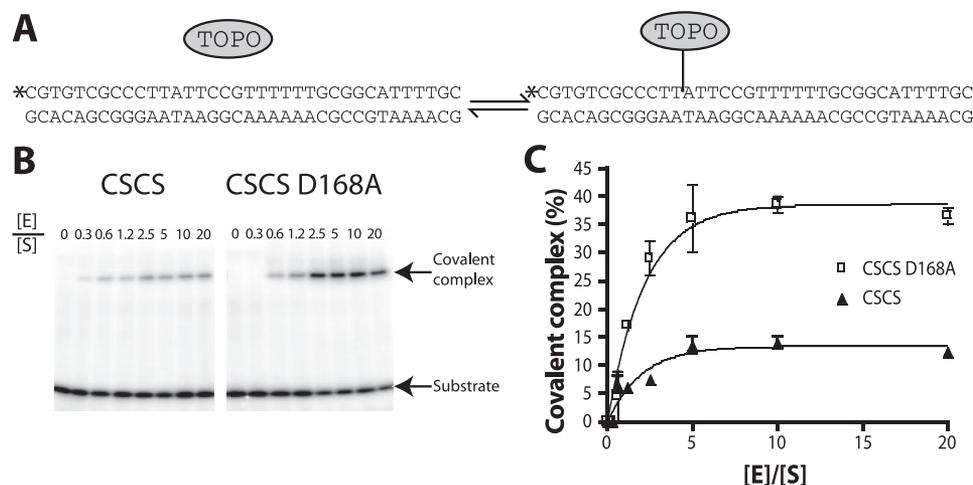


FIGURE 4. Assays of formation of covalent complex under equilibrium conditions on oligonucleotide substrates. A, diagram of the reaction and the substrates used. The DNA 5'-end labeled with  $^{32}\text{P}$  is indicated by the asterisk. B, autoradiogram illustrating protein titration experiments for CSCS and CSCS D168A. C, quantification of the titration experiments in B. Each reaction contained 8 pmol of the DNA substrate.

*Effects of Amino Acid Substitutions on Cleavage of Suicide Substrates*—The forward rate of DNA cleavage ( $k_{cl}$ ) can be monitored in isolation using “suicide substrates” (Fig. 3A). In these reactions, formation of the phosphotyrosine intermediate generates a short oligonucleotide that is released after cleavage, trapping the covalent complex. Sample time course studies comparing the CSCS enzyme and CSCS D168A and CSCS R206A are shown in Fig. 3B. To analyze the forward rate of cleavage, it is important to carry out reactions in the presence of saturating enzyme, so titration reactions were carried out initially to identify saturating conditions (data not shown). Examples of

quantitation of time course reactions are shown in Fig. 3, C and D, and rates are summarized in Table 1.

Topoisomerase derivatives with substitutions in the N-terminal domain and connecting  $\alpha$ -three helix (H39A, Q69A, and R80A) were reduced in rate 10–100-fold. Some of the catalytic domain contact residues were also strongly affected (CSCS with K133A, R206A, Y209A, and T266A), while CSCS K135A was nearly equal to CSCS. All of the catalytic site residues (CSCS with R130A, K167A, R223A, H265A, and Y274F) were severely reduced in activity as expected from previous work.

Residue Arg<sup>206</sup> was the only side chain contacting position +9 in the structure, allowing the basis of sequence specific recognition at this DNA position to be studied in more detail (Fig. 3D and Table 2). We found that substituting the +9 Gua residue with each of the other three bases reduced the forward rate of cleavage by the CSCS enzyme about 10–50-fold, indicating that this DNA position is recognized in a sequence specific fashion by the topoisomerase. When the Arg<sup>206</sup> side chain was truncated by alanine substitution, the reaction rate with the standard DNA substrate was reduced about 50-fold. Thus disrupting the +9 contact with either a DNA mutation or the R206A side chain truncation had quantitatively similar effects. However, when the CSCS R206A enzyme was tested on DNA substrates with substitutions at +9, no further reduction in rate was observed. Changes at +10 or +8 had little or no effect on the CSCS enzyme (<3-fold), and CSCS R206A was similarly unaffected. Both the CSCS topoisomerase and CSCS R206A were strongly reduced in activity by an Ade substitution at position +2 within the 5'-CCCTT-3' pentamer. This indicates that truncating the Arg<sup>206</sup> side chain resulted in a loss of ability to discriminate the base pair at position +9, but this was not a general loss of discrimination because CSCS R206A responded similarly to the CSCS topoisomerase to changes at positions +10, +8, and +2. Thus the functional analysis indicates that the Arg<sup>206</sup> contact is the basis of sequence discrimination at position +9.

Surprisingly, alanine substitutions at both Glu<sup>124</sup> and Asp<sup>168</sup> substantially increased the measured  $k_{cl}$ , a mutant phenotype

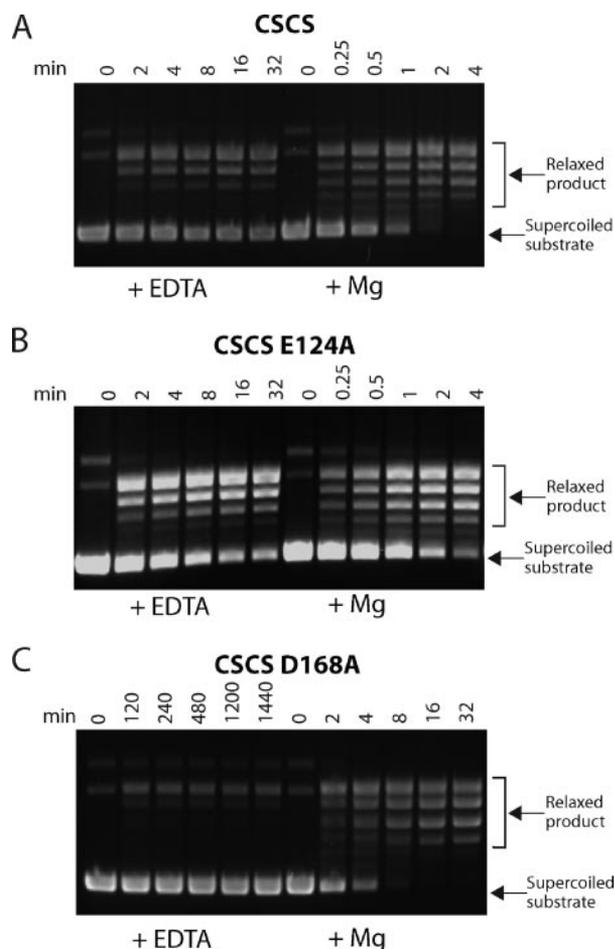


FIGURE 5. Effects of 5 mM  $\text{MgCl}_2$  or 2.5 mM EDTA on the relaxation activity of CSCS, CSCS D168A, and CSCS E124A. A, relaxation assays with CSCS. B, relaxation assays with CSCS E124A. C, relaxation assays with CSCS D168A. Note the different time scales used.

substitutions both carried out relaxation with rates similar to the CSCS enzyme. Of the DNA contact residues, the Q69A, R80A, K133A, and K135A substitutions all showed notably longer half-times for DNA relaxation.

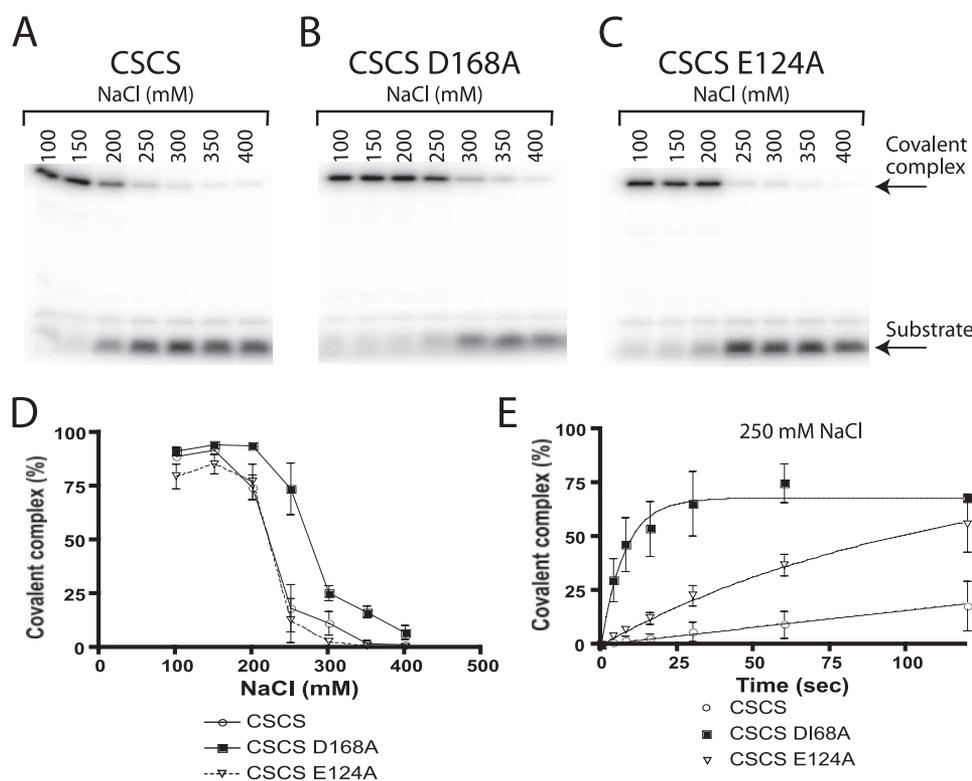


FIGURE 6. Effects of increased NaCl concentration on the activity of CSCS (A), CSCS D168A (B), and CSCS E124A (C). Reaction times were chosen to allow sensitive detection of small changes in activity, and were: 30 s for CSCS, 5 s for CSCS D168A, and 10 s for CSCS E124A. D, comparison of the reaction efficiencies at different NaCl concentrations. E, cleavage rates at 250 mM NaCl. Rates (1/s) measured by non-linear regression were  $4.5 \times 10^{-5}$  for CSCS,  $1.3 \times 10^{-1}$  for CSCS D168A, and  $9.2 \times 10^{-3}$  for E124A. Because of the very slow rate the value for CSCS had high uncertainty.

not previously reported despite the wealth of mutagenesis studies for the related vaccinia enzyme. For CSCS D168A, the apparent forward rate of cleavage was increased by more than 10-fold. The E124A substitution increased the forward cleavage rate 5-fold. Curiously, the E124Q substitution slightly slowed the cleavage rate. We discuss the possible explanations for these observations below.

**Effects of Mutants Assayed on Equilibrium Cleavage Substrates**—The position of the equilibrium defined by the rates of covalent complex formation and religation ( $k_{cl}$ ) can be measured on equilibrium oligonucleotide substrates. These substrates contain the 5'-CCCTT-3' core pentamer in the center of a 36-mer oligonucleotide. After transesterification to form the 3'-phosphotyrosine intermediate, all the strands remain annealed because of the longer DNA lengths (Fig. 4A). This is in contrast to the suicide substrate, where the free portion of the cleaved strand is released (Fig. 3A). Consequently, in the equilibrium substrate, the 5'-hydroxyl of the cleaved strand is available for religation. The cleavage and religation reactions are quite fast on these substrates, achieving equilibrium in most cases within 4 s (data not shown). Thus the fraction of the DNA in the covalent complex at times  $>4$  s reports the position of the cleavage-religation equilibrium. In addition, the religation rates ( $k_{rel}$ ) can be calculated from the measured forward rates of cleavage ( $k_{cl}$ ) and the position of the cleavage religation equilibrium ( $K_{cl}$ ) using the relationship  $K_{cl} = k_{cl}/k_{rel}$ . These values are shown in the right column in Table 1.

The amounts of enzyme required for saturation were first determined by titration as shown in Fig. 4, B and C. Table 1 summarizes the fraction of DNA molecules in the covalent complex at equilibrium for all the topoisomerase derivatives tested. For the CSCS reference enzyme, the fraction of substrate molecules in the covalent complex at equilibrium is 0.13. For CSCS with several of the active site substitutions, the equilibrium position was not reached by  $>24$  h (R130A, K167A, and R223A), or no covalent complex formation was detected (CSCS Y274F). CSCS H265A reached equilibrium after 4 min. At equilibrium, the percentage of substrate in the covalent complex was 0.35, consistent with a more severe defect for the CSCS H265A enzyme in religation than in the forward cleavage step, as suggested in previous work in the vaccinia system (17, 20).

For several of the enzymes with substitutions in DNA contact residues, the position of the equilibrium  $K_{cl}$  was changed, so that at the steady state less of the DNA was in the covalent complex (Table 1).

This is consistent with the idea that these protein-DNA contacts are more important for activating the initial cleavage reaction than for religation. Relatively strong effects were seen for CSCS with Q69A, R80A, K133A, R206A, Y209A, and T266A. Weaker but possibly significant reductions in ratio were seen at CSCS with K135A and H39A.

For CSCS D168A, the fraction of substrate molecules in the covalent complex was increased to 0.36. The Asp<sup>168</sup> side chain is positioned on the side of the active site near His<sup>265</sup> and close to the inferred position of the 5'-OH group that participates in religation. Thus Asp<sup>168</sup> is implicated by this data in religation as well.

For the Glu<sup>124</sup> substitutions, the two changes studied had different effects. CSCS E124Q was slightly altered, so that only about half as much of the substrate was in the covalent complex at equilibrium (0.06). For CSCS E124A, there was no significant change compared with the CSCS reference enzyme.

**Effects of 5 mM MgCl<sub>2</sub> on the Activity of CSCS, CSCS D168A, and CSCS E124A**—A possible mechanism for the reduction in relaxation rate with CSCS D168A is that it may be defective in product release. In the relaxation assay, the plasmid DNA substrate is in excess, so each topoisomerase molecule must undergo several cycles of DNA cleavage, religation, and product release to complete relaxation of the plasmid population. Studies of the vaccinia topoisomerase have shown that relaxation reactions carried out under the conditions in Fig. 2 are limited at the product release step. However, addition of 5 mM

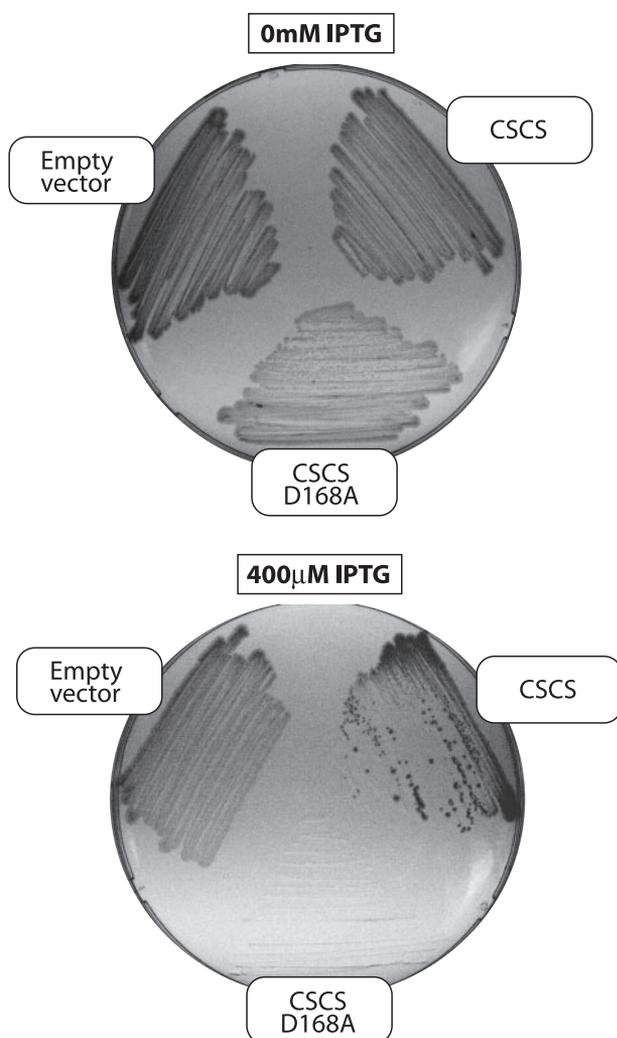


FIGURE 7. Toxicity of the D168A mutant assayed in *E. coli*. Each panel shows a bacterial plate streaked with *E. coli* expressing the indicated topoisomerase derivatives. The amounts of IPTG inducer cast into the plates is shown above each. The bacterial strains contained the indicated expression vectors.

$\text{MgCl}_2$  accelerates release without affecting the cleavage rate  $k_{cl}$  (30). We thus compared reactions containing 5 mM  $\text{MgCl}_2$  to reactions containing 2.5 mM EDTA (Fig. 5).

For the CSCS enzyme, we found that the time to 50% relaxation was reduced from 12 min in EDTA to 0.75 min in the presence of  $\text{MgCl}_2$ , a reduction of 16-fold. For CSCS D168A, the half-time was unmeasurable in 2.5 mM EDTA because a portion of the substrate became relaxed quickly but no further relaxation took place even at long incubation times. Addition of 5 mM  $\text{MgCl}_2$  allowed complete relaxation of the substrate, with a half-time of 2.4 min, representing an increase of at least 600-fold. For CSCS E124A, the half-time went from 9.3 min in 2.5 mM EDTA to 1 min in 5 mM  $\text{MgCl}_2$ , roughly paralleling the acceleration seen with the CSCS reference enzyme. Thus the time necessary to relax half the substrate was reduced for CSCS, CSCS D168A, and CSCS E124A in 5 mM  $\text{MgCl}_2$ , and the change was comparatively greater for CSCS D168A. This supported the hypothesis that product release is limiting, and that the barrier is comparatively greater with CSCS D168A.

*Effects of NaCl Concentration on the Activity of CSCS, CSCS D168A, and CSCS E124A*—We next investigated whether CSCS D168A binds DNA more tightly than the CSCS reference enzyme. Previous studies of the vaccinia topoisomerase have shown that DNA binding affinity can be probed in the single cycle cleavage assay by titration of NaCl in reaction mixtures. The increased salt concentration diminishes DNA affinity by ionic screening without affecting  $k_{cl}$  (30), so that altered DNA affinity results in slower covalent complex formation.

Comparison of covalent complex formation for CSCS, CSCS D168A, and CSCS E124A in the presence of different NaCl concentrations showed that CSCS D168A was less sensitive to increased NaCl concentrations (Fig. 6). To carry out this study, reaction times were adjusted for each enzyme to maximize the sensitivity of detecting small differences. Titration of NaCl revealed that all enzymes showed a progressive reduction in rate above 250 mM NaCl, but the decline was faster for CSCS and CSCS E124A (Fig. 6, A–C, results are plotted in Fig. 6D). Reaction rates were measured at 250 mM NaCl, and found to be reduced (compared with 100 mM NaCl) by 43-fold for CSCS, 10.5-fold for CSCS E124A, and 1.9-fold for CSCS D168A (Fig. 6E). These data support the idea that CSCS D168A is considerably increased in DNA binding affinity compared with CSCS, while CSCS E124A may be slightly increased.

*Toxicity of the D168A Topoisomerase in E. coli*—We also observed that the CSCS D168A was more toxic to *E. coli* cells during overexpression than the unmodified CSCS enzyme (Fig. 7). To document this, *E. coli* cells transformed with topoisomerase expression vectors were streaked on plates lacking IPTG,<sup>3</sup> and so not inducing topoisomerase expression, or plates containing 400  $\mu\text{M}$  IPTG, which induce topoisomerase expression to high levels. After overnight incubation at 37 °C, no growth was seen for cells containing the D168A mutant in the presence of 400  $\mu\text{M}$  IPTG. Growth was seen, however, in the presence of CSCS D168A in the absence of IPTG. Cells expressing the CSCS topoisomerase, or containing the empty expression vector, grew in both the presence and absence of 400  $\mu\text{M}$  IPTG. Expression of the CSCS did have some effect on cells, since there was less growth than for cells containing the empty expression vector, and after prolonged storage of plates many of the transformed cells died (data not shown). For the case of CSCS E124A and CSCS E124Q, there was no reduction of growth of *E. coli* under inducing conditions (data not shown). Active site substitutions also abrogated the toxicity (data not shown). Comparison of cells expressing the CSCS topoisomerase with cells expressing the fully wild-type enzyme showed that the wild type was slightly more toxic (data not shown), consistent with the CSCS substitutions reducing the enzyme activity slightly. We did not attempt to study D168A or E124A in the fully wild-type background (*i.e.* Cys at 100 and 211) because of the expected increase in toxicity. In summary, the most notable finding was that truncating the Asp<sup>168</sup> side chain by the alanine substitution resulted in increased toxicity of topoisomerase expression in *E. coli*.

<sup>3</sup> The abbreviation used is: IPTG, isopropyl-1-thio- $\beta$ -D-galactopyranoside.

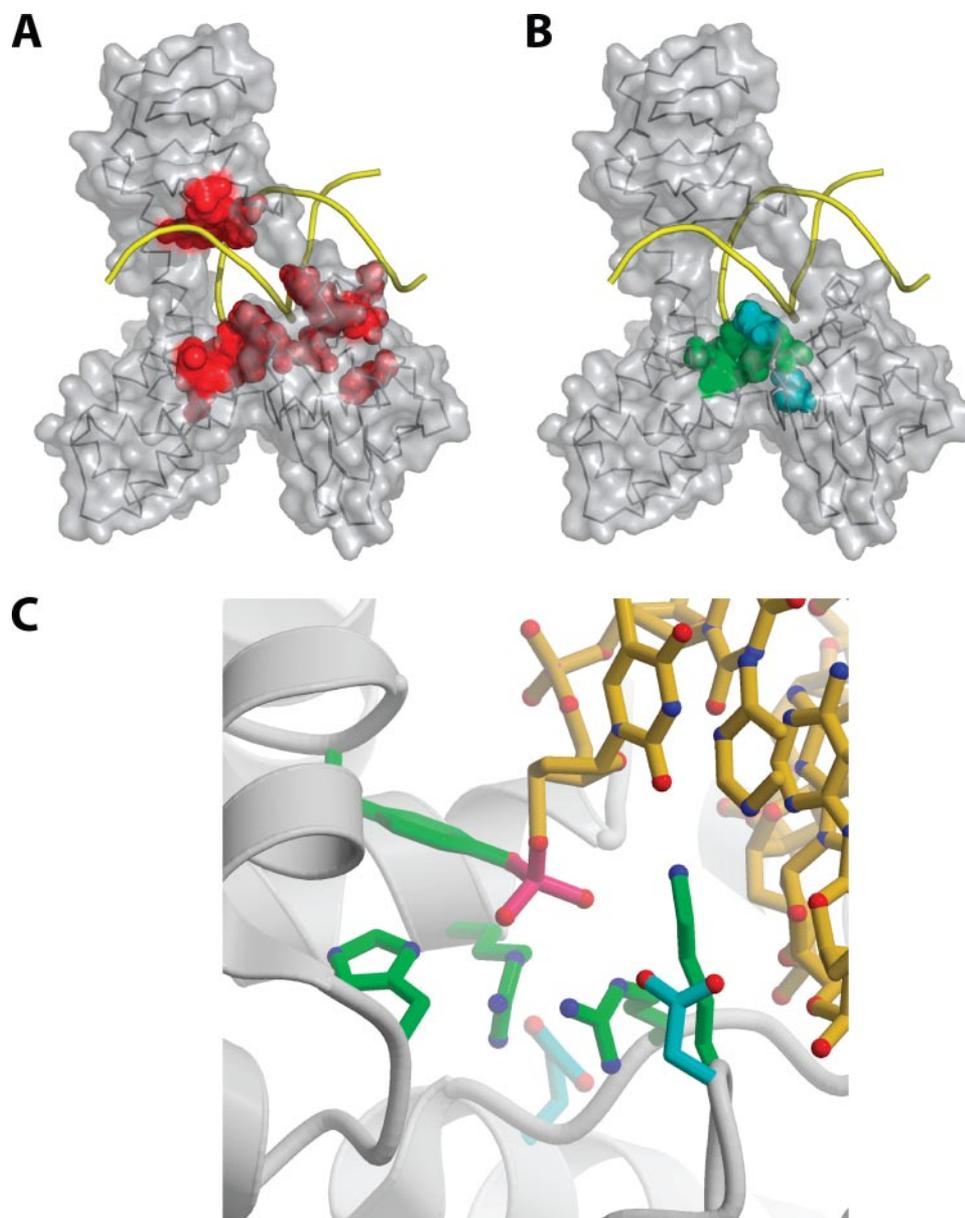


FIGURE 8. Positions of amino acid substitutions influencing topoisomerase function mapped on the topoisomerase-DNA structure. *A*, positions of substitutions affecting the forward rate of cleavage on suicide substrates are shown in *red*. *B*, positions of side chains, Glu<sup>124</sup> and Asp<sup>168</sup>, that when substituted with alanine boost the reaction rate. Glu<sup>124</sup> and Asp<sup>168</sup> are shown in *blue*, the active site residues are shown in *green*. *C*, close-up view of the active site, showing the relationship of Asp<sup>168</sup> and Glu<sup>124</sup> to the catalytic residues.

## DISCUSSION

The phenotypes of mutant topoisomerase derivatives analyzed in this study are summarized on the topoisomerase/DNA complex structure in Fig. 8 together with mutants previously analyzed in the vaccinia topoisomerase system (6, 12, 15, 17–20, 29, 31–33) (summarized in supplemental Table S1). Positions of substitutions that reduced the forward rate of cleavage  $k_{cl}$  are shown in *red* in Fig. 8*A*. Substitutions that increased  $k_{cl}$  are shown in *blue* in Fig. 8*B*. Amino acid side chains important in the forward cleavage reaction line the protein/DNA interface, extending from DNA residues +9 to at least –1. These amino acids include the catalytic site residues and amino acids within the central cavity of the enzyme. Most of the residues implicated as making sequence specific contacts from analysis of the

x-ray structures had detectable phenotypes when substituted with alanine. Studies of alanine substitutions of Arg<sup>206</sup>, Asp<sup>168</sup>, and Glu<sup>124</sup> were particularly informative and are discussed further below.

The importance of the contact between Arg<sup>206</sup> and Gua at +9 could be documented by a “loss-of-discrimination” experiment. Substituting the Arg<sup>206</sup> side chain with alanine resulted in a reduction in the forward rate of cleavage. Substitutions at position +9 in the DNA similarly diminished cleavage by the CSCS topoisomerase. However, when CSCS R206A was tested in the presence of substitutions at +9, no further reduction in rate was found. Both the CSCS enzyme and CSCS R206A remained capable of discriminating base pair substitutions at position +2. Thus the selective loss of discrimination with CSCS R206A at the +9 base pair implicates the R206 contact as the basis of specific recognition.

CSCS D168A and CSCS E124A showed a novel phenotype, which was an acceleration of the apparent forward rate of cleavage on the suicide substrate. Both Asp<sup>168</sup> and Glu<sup>124</sup> lie at the margins of the active site. Asp<sup>168</sup> lies near the protein surface close to the inferred binding site for the 5'-oxygen that acts as the nucleophile for religation. Glu<sup>124</sup> is buried in the protein interior. Both of these residues are strictly conserved in the poxvirus topoisomerases, though not universally conserved in the other type IB enzymes. The conservation suggests that these side chains carry out

important functions in the poxvirus enzymes, but that they are probably not strictly required for catalysis.

Studies of CSCS D168A were particularly informative. CSCS D168A showed a reduction in the rate of DNA relaxation under conditions requiring multiple cycles of DNA relaxation and product release. Titration of NaCl in forward cleavage assays indicated that CSCS D168A has an increased affinity for DNA. Accelerating product release in the relaxation assay by addition of 5 mM MgCl<sub>2</sub> resulted in a dramatic increase in rate for CSCS D168A, consistent with the idea that tighter DNA binding led to impaired product release. One simple explanation for the increased DNA affinity would be that the acidic D168 side chain normally engages in an electrostatic repulsion with the negatively charged phosphates in the substrate DNA, so that remov-

ing the side chain increased the affinity. The D168A substitution could have increased the forward rate of cleavage by several means. Single cycle cleavage assays were carried out in the presence of saturating topoisomerase, so tighter DNA binding alone is not likely to be responsible for the faster rate. The D168A substitution might increase binding in a conformation required for cleavage, or act directly at the chemical step to accelerate catalysis. Assays using the equilibrium cleavage substrate revealed a greater proportion of covalent complexes at steady state for CSCS D168A than for wild-type CSCS, indicating that the forward rate of cleavage was increased more than the religation rate (13-fold for  $k_{cl}$  versus 4.6-fold for  $k_{rel}$ ). In the structure, the Asp<sup>168</sup> side chain lies near the expected position of the 5'-hydroxyl group that is the nucleophile for religation, close to the His<sup>265</sup> side chain that is also implicated in religation by data in Table 1 and previously published studies (6, 12, 15, 17, 18, 20, 32). The Asp<sup>168</sup> side chain may normally help remove a proton from the 5'-hydroxyl group that is the nucleophile for religation, and conversely the D168A substitution may make the environment more acidic, thereby promoting cleavage. More broadly, the topoisomerase must maintain a delicate balance between cleavage, allowing release of DNA supercoils, and religation, which restores DNA continuity but prevents further supercoil release (15). This work implicates Asp<sup>168</sup> as a key element of the active site that modulates and optimizes DNA affinity and the cleavage and religation efficiencies.

The role of the Glu<sup>124</sup> side chain is less clear. Substitution with alanine accelerated the rate of cleavage on the suicide substrate 5-fold, but the E124Q substitution had no effect. For CSCS E124A, the position of the cleavage-religation equilibrium was unchanged, implying that the religation rate was accelerated as much as cleavage. CSCS E124A showed slightly reduced sensitivity to 250 mM NaCl, indicating possible tighter DNA binding, though this was not pronounced enough to inhibit religation by impairing product release. Possibly the E124A substitution enhanced binding in a conformation promoting cleavage or increased the catalytic rate, though it would require further experiments to clarify the mechanism.

A goal of studies of the smallpox topoisomerase has been to develop topoisomerase poisons that might be used as therapy for poxvirus infection. The finding that CSCS D168A is toxic in *E. coli* may be helpful in this regard. The D168 and H265 side chains are near one another, specifying a region of the enzyme surface important for religation. Potentially small molecules might be identified that bind to this region and so interfere with religation and thus could serve as poisons of the poxvirus topoisomerases. The smallpox topoisomerase/DNA complex x-ray structures (22) provide templates for possible design of antiviral molecules based on this idea.

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