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p-Thiophenylalanine-Induced DNA Cleavage and Religation Activity of a Modified Vaccinia Topoisomerase IB

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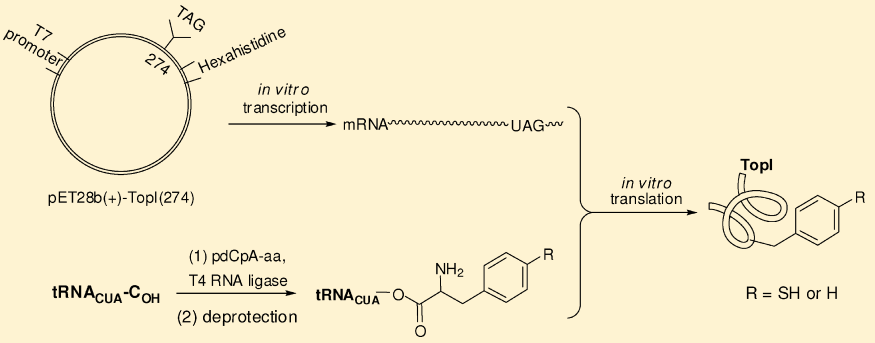
Supporting Information



ABSTRACT: Vaccinia DNA topoisomerase IB is the smallest of the type IB topoisomerases. Because of its small size (314 amino acids) and target site specificity (5′(C/T)CCTTp$^{\downarrow}$ sites), it constitutes an excellent model for studying the interaction of type IB enzymes with duplex DNA. In this study, *p*-thiophenylalanine was incorporated into the enzyme active site (position 274) by *in vitro* translation in the presence of a chemically misacylated tRNA. The modification, which resulted in replacement of the nucleophilic tyrosine OH group with SH, retained DNA topoisomerase activity and did not alter the DNA cleavage site. However, the modified topoisomerase effected relaxation of supercoiled plasmid DNA at a rate about 16-fold slower than the wild-type enzyme. The thiophenylalanine-induced DNA cleavage rate ($k_{cl} = 1 \times 10^{-4}$ s^{-1}) was 30 times lower than for the wild-type enzyme ($k_{cl} = 3 \times 10^{-3}$ s^{-1}). In contrast, thiophenylalanine-induced DNA religation was faster than that of the wild-type enzyme. We propose that the change in kinetics reflects the difference in bond energies between the O−P and S−P bonds being formed and broken in the reactions catalyzed by the wild-type and modified enzymes. We also studied the effect of adding Mg^{2+} and Mn^{2+} to the wild-type and modified topoisomerases I. Divalent metal ions such as Mg^{2+} and Mn^{2+} increased DNA relaxation activity of the wild-type and modified enzymes. However, the pattern of increases failed to support the possibility that metal ion−heteroatom interaction is required for catalysis.

DNA topoisomerases, a class of enzymes involved in DNA replication, chromosome segregation, genetic recombination, and transcription, alter the supercoiling of double-stranded DNA by transiently cleaving and rejoining DNA strands.[1−3] Type I enzymes cleave one strand of the duplex and allow passage of the intact strand through the nick. In contrast, type II enzymes cut both strands of the DNA through which another DNA segment is passed.[4]

Vaccinia virus topoisomerase IB, a 314-amino acid monomer, is the smallest topoisomerase IB enzyme.[5] This viral enzyme is distinguished from its nuclear counterparts by its sequence specificity in strand cleavage. Vaccinia topoisomerase IB binds duplex DNA and forms a covalent adduct at sites containing the sequence 5′(C/T)CCTTp$^{\downarrow}$ in the scissile strand.[6−8] During this process, the topoisomerase utilizes a tyrosine residue (Tyr274 in vaccinia topoisomerase I) in a nucleophilic attack on a phosphate ester in the DNA backbone in order to produce transient DNA strand scission. The DNA phosphodiester linkage is restored by a process that is nominally the reversal of this transesterification reaction; it results in resealing of the cleaved DNA backbone with concomitant release of the enzyme from the DNA.[9] Therefore, modifying Tyr 274 can potentially provide useful information concerning the catalytic mechanism of vaccinia topoisomerase IB.

The transesterification reaction has been characterized using modified DNA substrates, such as those containing methylphosphonates[10−12] or 2′,5′-phosphate ester linkages in proximity to cleavage sites.[13−15] Henningfeld et al. have also modified the 5′-OH group at the site of DNA strand scission to study the religation reaction.[16] They reported that a modified acceptor oligonucleotide containing an SH group at the 5′-end of the cleaved DNA gave the expected ligation product. The

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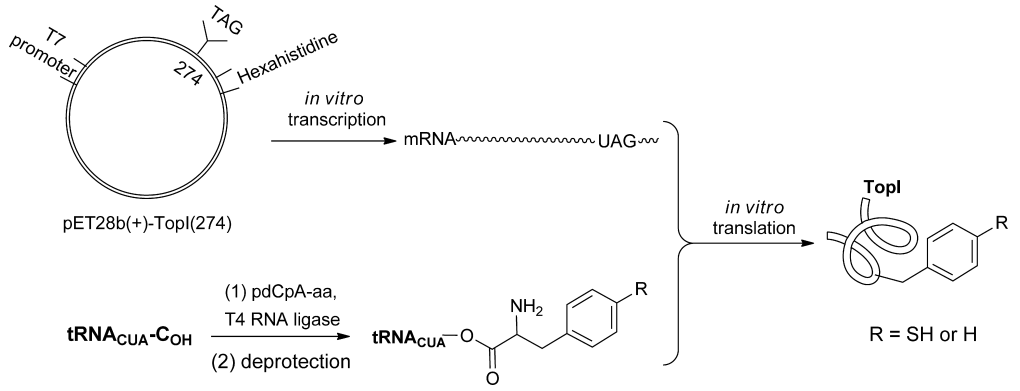
Scheme 1. Strategy Employed for Incorporation of Tyrosine Analogues into Vaccinia Topoisomerase I at Position 274



yield was 13% relative to the unmodified DNA oligonucleotide acceptor.

Recent efforts in our laboratory have involved modification of the vaccinia topoisomerase IB at Tyr274 to incorporate tyrosine analogues with altered steric, electronic, and regiochemical features.[17] This was accomplished by employing suppressor tRNAs activated with the tyrosine analogues of interest in an *in vitro* protein synthesizing system programmed with topoisomerase I mRNA containing a nonsense codon (UAG) at position 274 (Scheme 1). Meta-substituted analogues of Tyr274 reduced the rate of DNA cleavage without affecting the rate of religation.[17,18] Several para-substituted anologues (e.g., −SH, −NH_2, and −I) were prepared and evaluated but found to be inactive.[17] However, in more recent work, it was found that the conditions employed previously for deprotection of the *n*-butyl disulfide protecting group in topoisomerase I containing *p*-thiophenylalanine (1 mM dithiothreitol) may not have been sufficient to effect complete deprotection.

Accordingly, in the present study, we removed the butyl disulfide protecting group of thiophenylalanine with 100 mM DTT[19] at the tRNA level to afford thiophenylalanyl-tRNA. Deprotected thiophenylalanine was then incorporated into the active site of vaccinia topoisomerase IB.[17] The modified topoisomerase I prepared in this fashion was found to be catalytically competent for reversible DNA strand scission. In particular, the thiophenylalanine-induced DNA cleavage rate by this modified enzyme was 30 times lower than for the wild-type enzyme. In contrast, the thiophenylalanine-induced religation rate was greater than that of the wild-type topoisomerase I.

MATERIALS AND METHODS

General Materials and Methods. Ni-NTA agarose was obtained from Qiagen Inc. (Valencia, CA). DNA oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). Ultrapure agarose was obtained from Bethesda Research Laboratories (Bethesda, MD). Ammonium persulfate, acrylamide, *N*,*N*′-methylenebis(acrylamide), manganese chloride, acetic acid, EDTA, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phospho(enol)-pyruvate, *Escherichia coli* tRNA, isopropyl β-D-thiogalactopyranoside (IPTG), ATP, GTP, CTP, UTP, cAMP, amino acids, rifampicin, and formamide were obtained from Sigma-Aldrich (St. Louis, MO). Tris and SDS were obtained from Bio-Rad Laboratories (Hercules, CA). [^{35}S]Methionine (1000 Ci/mmol, 10 μCi/μL) and γ-^{32}P-ATP (10 μCi/μL) were purchased from PerkinElmer Inc. (Boston, MA). Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. (Indianapolis, IN). T4 RNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs Inc. (Ipswich, MA).

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a PerkinElmer Lamdba 20 UV/vis spectrometer.

Vaccinia topoisomerase IB analogues modified at position 274 with thiophenylalanine and phenylalanine were prepared using the general strategy shown in Scheme 1.[20−31]

***N*-(6-Nitroveratryloxycarbonyl)thiophenylalanine *n*-Butyl Disulfide (2).** A solution of 320 mg (1.12 mmol) of thiophenylalanine *n*-butyl disulfide (**1**)[32] in 10 mL of water was added 188 mg (2.24 mmol) of $NaHCO_3$ followed by 370 mg (1.34 mmol) of 6-nitroveratryl chloroformate[33] in 10 mL of 1,4-dioxane. The reaction mixture was stirred at room temperature for 3 h and then acidified with 1 N $NaHSO_4$ to pH 4. The reaction mixture was extracted with two 25 mL portions of ethyl acetate. The organic layer was dried (Na_2SO_4), and the solvent was concentrated under diminished pressure. The residue was applied to a silica gel column (24 × 1.2 cm). Elution with 50:1 ethyl acetate−acetic acid afforded **2** as a yellow foam: yield 330 mg (56%). ^{1}H NMR (500 MHz, DMSO-d_6) δ: 0.88 (m, 3H), 1.39 (m, 2H), 1.65 (m, 2H), 2.71 (m, 2H), 3.09−3.22 (m, 2H), 3.91 (s, 3H), 3.94 (s, 3H), 4.67 (m, 1H), 5.38 (m, 1H), 5.50 (m, 2H), 6.92 (s, 1H), 7.14 (d, 2H), 7.46 (d, 2H) and 7.69 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 13.7, 21.6, 31.3, 37.0, 38.9, 54.5, 56.3, 56.4, 64.0, 108.1, 109.9, 127.5, 127.7, 128.2, 129.0, 129.8, 134.0, 136.9, 139.6, 148.1, 153.6, 155.4, and 175.3. Mass spectrum (FAB), *m*/*z* 525.1359 $(M + H)^+$ ($C_{23}H_{29}N_2O_8S_2$ requires 525.1365).

***N*-(6-Nitroveratryloxycarbonyl)thiophenylalanine *n*-Butyl Disulfide Cyanomethyl Ester (3).**[34] A solution of 300 mg (0.57 mmol) *N*-(6-nitroveratryloxycarbonyl)-thiophenylalanine *n*-butyl disulfide in 15 mL of anhydrous acetonitrile was added 285 mg (2.82 mmol) of Et_3N, followed by 367 mg (4.86 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 16 h, then 35 mL of ethyl acetate was added. The organic layer was washed with 25 mL of saturated $NaHCO_3$ followed by 25 mL of saturated aqueous NaCl. The organic layer was dried over Na_2SO_4 and concentrated under diminished pressure. The residue was applied to a silica gel column (24 × 1.2 cm). Elution with 1:1 ethyl acetate−hexanes afforded **3** as a yellow foam: yield 184 mg (57%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 0.92 (m, 3H), 1.41

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(m, 2H), 1.66 (m, 2H), 2.74 (m, 2H), 3.15 (m, 2H), 3.94 (s, 3H), 3.96 (s, 3H), 4.68−4.85 (m, 3H), 5.28 (q, 1H), 5.50 (m, 2H), 6.92 (d, 1H), 7.12 (d, 2H), 7.50 (q, 2H) and 7.72 (d, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 13.6, 21.5, 30.8, 37.0, 38.6, 49.0, 54.5, 56.3, 56.4, 64.1, 108.1, 109.9, 113.6, 127.4, 127.5, 127.6, 127.7, 129.6, 133.1, 137.2, 139.6, 148.1, 153.6, 155.1, and 170.2. Mass spectrum (FAB), m/z 564.1481 $(M + H)^+$ ($C_{25}H_{30}N_3O_8S_2$ requires 564.1474).

***N*-(6-Nitroveratryloxycarbonyl)thiophenylalanyl-pdCpA *n*-Butyl Disulfide (4).** A solution of 14.8 mg (26.3 μmol) of *N*-(6-nitroveratryloxycarbonyl)thiophenylalanine *n*-butyl disulfide cyanomethyl ester in 50 μL of anhydrous DMF was added 3.2 mg (2.35 μmol) of the tetra-*n*-butylammonium salt of pdCpA[35] in 50 μL of anhydrous DMF, followed by 10 μL of triethylamine. The reaction mixture was kept in the dark and stirred at room temperature for 36 h; the course of the reaction was monitored by reversed phase HPLC. The reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN−50 mM NH_4OAc, pH 4.5, and purified on a C_{18} reversed phase HPLC column (10 μm, 250 × 10 mm). The column was washed with a linear gradient of 1 → 63% CH_3CN−50 mmol NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min. The product (t_R 35.7 min) was lyophilized to give **4** as a yellow foam: yield 1.2 mg (45%). Mass spectrum (FAB), m/z 1141.2210 $(M - H)^-$ ($C_{42}H_{51}N_{10}O_{20}P_2S_2$ requires 1141.2198).

Ligation of Suppressor tRNA-C_{OH} with Thiophenylalanyl-pdCpA and Phenylalanyl-pdCpA. Suppressor tRNA aminoacylation was carried out in 100 μL (total volume) of 100 mM Hepes buffer, pH 7.5, containing 0.5 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} units of protected aminoacyl-pdCpA (5−10-fold molar excess), 15% DMSO, and 200 units of T4 RNA ligase. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The reaction mixture was incubated at −20 °C for 30 min and then centrifuged at 14000*g* at 4 °C for 30 min. The supernatant was carefully decanted, and the tRNA pellet was washed with 100 μL of 70% ethanol and dissolved in 100 μL of RNase free H_2O. The efficiency of ligation was estimated by 8% denaturing PAGE (pH 5.2).[17,36] The *n*-butyl disulfide protecting group of *N*-(6-nitroveratryloxycarbonyl)thiophenylalanyl-tRNA *n*-butyl disulfide was removed using 100 mM DTT at 0 °C for 1 h.[19] The reaction was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The reaction mixture was incubated at −20 °C for 30 min and centrifuged at 14000*g* at 4 °C for 30 min, and then the supernatant was carefully decanted. The tRNA pellet was washed with 100 μL of 70% aqueous ethanol and dissolved in 30 μL of RNase free H_2O. The NVOC-protected aminoacyl-tRNA was cooled to 2 °C and irradiated with a 500 W mercury−xenon lamp for 5 min.[30,33] After irradiation, deblocked aminoacylated suppressor tRNAs were using *in vitro* suppression experiments without further purification. For deprotection of the *N*-(4-pentenoyl)-phenylalanyl-tRNA, the pentenoyl group was removed by treatment with 5 mM I_2 at room temperature for 10 min.[30,31] Then the reaction was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The mixture was incubated at −20 °C for 30 min and then centrifuged at 14000*g* at 4 °C for 30 min. The supernatant was then carefully decanted. The tRNA pellet was washed with 100 μL of 70% ethanol and dissolved in 30 μL of RNase free H_2O.

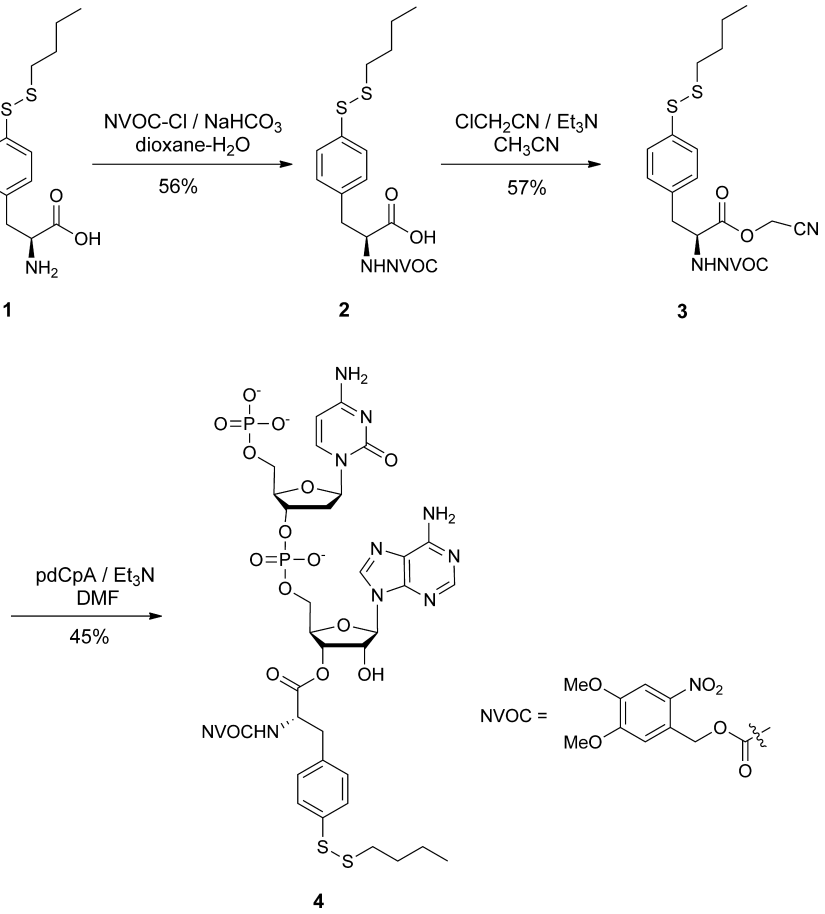
***In Vitro* Translation of Vaccinia Topoisomerase IB.[37]** The mutant vaccinia topoisomerase IB plasmid (TAG at position 274[18]) was added to the protein synthesis system. The reaction mixture (200 μL total volume) contained 15 μg of plasmid DNA, 80 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM maganesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM IPTG, 20 mM ATP and GTP, 5 mM CTP and UTP and 4 mM cAMP), 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-L-methionine, 10 μg/μL rifampicin, 60 μg of deprotected misacylated $tRNA_{CUA}$, and 60 μL of S-30 extract from *E. coli* strain BL21(DE3). The mixture was incubated at 37 °C for 45 min. Plasmid DNA containing the gene for wild-type topoisomerase I was used as the positive control, and an abbreviated tRNA (tRNA-C_{OH}) lacking any amino acid was used as the negative control. An aliquot containing 2 μL of reaction mixture was removed, treated with 5 μL of loading buffer, and heated at 90 °C for 2 min. This was analyzed by 16.5% SDS-PAGE at 100 V for 2 h. The analogues of vaccinia topoisomerase IB (containing a C-terminal hexahistidine fusion peptide[18]) were purified by Ni-NTA chromatography,[38] as described below. SDS-PAGE electrophoresis was used to determine protein concentration.[17]

Purification of Analogues of Topoisomerase IB. The analogues of vaccinia topoisomerase IB containing a C-terminal hexahistidine were purified by Ni-NTA chromatography.[38] The *in vitro* translation reaction mixture (200 μL) was diluted with 600 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 20 mM imidazole, and mixed gently with 200 μL of a 50% slurry of Ni-NTA resin at 4 °C for 2 h. Then the mixture was loaded on a column and washed with 500 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 50 mM imidazole. Finally, the pure vaccinia topoisomerase IB was eluted with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 150 mM imidazole. Aliquots of each fraction were analyzed by 16.5% SDS-PAGE.

Plasmid DNA Relaxation Assay. A reaction mixture (10 μL total volume) containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 250 ng of supercoiled pSP64 plasmid DNA, and 1 ng of topoisomerase IB was incubated at 37 °C for 20 min and quenched by addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis.[17] After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and then rerun for 30 min to separate the relaxed (form IV) and nicked (form II) DNAs.[39] Gels were visualized using UV light.

Concentration Dependence of DNA Relaxation Assay. The relaxation assay was carried out in a 10 μL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM EDTA, 250 ng of supercoiled pSP64 plasmid DNA and topoisomerase IB (0.13, 0.25, 0.5, and 1.0 ng of wild-type or 0.5, 1.0, 1.5, and 2.0 ng of modified topoisomerase). Reactions were incubated at 37 °C for 30 min and quenched by addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis.[17] After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and then rerun for

Scheme 2. Synthesis of *N*-(6-Nitroveratryloxycarbonyl)thiophenylalanyl-pdCpA *n*-Butyl Disulfide



30 min to separate the relaxed (form IV) and nicked (form II) DNAs.[39] Gels were visualized using UV light, and the extent of reaction (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase IB) was quantified utilizing ImageQuant version 5.2 software.

Kinetics of DNA Relaxation by Vaccinia Topoisomerase IB. Supercoiled plasmid DNA (250 ng) was treated with 1 ng of vaccinia topoisomerase I in a 10 μL reaction mixture containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 2.5 mM EDTA. The reaction mixture was incubated at 37 °C and quenched at predetermined times by the addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis.[17] After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and then rerun for 30 min to separate the relaxed (form IV) and nicked (form II) DNAs.[39] Gels were visualized using UV light, and the extent of reaction (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase IB) was quantified by utilizing ImageQuant version 5.2 software. The relaxation rate constants (k_r) were determined by fitting the data to the equation [100 − % relaxation] = $100e^{-kt}$.

Assay for pH Dependence of DNA Relaxation. Relaxation assays were performed at different pH values using the following buffers: sodium acetate, pH 4.0−6.5; Tris-HCl, pH 7.0−10.0. A reaction mixture (10 μL total volume) containing 50 mM buffer, 100 mM NaCl, 2.5 mM EDTA, 250 ng of supercoiled pSP64 plasmid DNA, and topoisomerase IB (1 ng of wild-type or 1.5 ng of modified enzyme) was incubated at 37 °C for 20 min and quenched by the addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue). The reaction mixture was analyzed by 1% agarose gel electrophoresis.[17] After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and then rerun for 30 min to separate the relaxed (form IV) and nicked (form II) DNAs.[39] Gels were visualized under UV light, and the extent of reaction (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase IB) was quantified by utilizing ImageQuant version 5.2 software.

Effect of the Thiophenylalanine-Modified Topoisomerase Analogue on DNA Single-Turnover Cleavage. Oligonucleotide (5′-dCGT GTC GCC CTT ATT CCC-3′) was ^{32}P-end labeled at the 5′-end with γ-^{32}P-ATP using T4 polynucleotide kinase in a 10 μL reaction mixture containing 200 pmol of oligonucleotide, 50 pmol of γ-^{32}P-ATP, and 10 U of T4 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 1 h and then was purified on a 20% acrylamide gel. The cleavage reaction mixtures (40 μL) containing 50 mM Tris-HCl (pH 7.5 for wild-type and pH 7.0 for the thiophenylalanine analogue), 40 fmol of 5′-^{32}P-labeled 18-mer/30-mer DNA substrate (depicted in Figure 5A with the cleavage site indicated by the arrow), and *in vitro* translated topoisomerase IB (20 ng of wild-type, 20 or 40 ng of modified enzyme) were incubated at 37 °C. The reactions were

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quenched with 1% aqueous SDS at 3, 10, 30, 60, 90, and 120 min. The reaction mixture was treated with 3 volumes of ethanol and precipitated to remove SDS. Then 10 μL of 50% formamide was added, and the reaction mixture was heat-denatured and then analyzed by electrophoresis through a 20% polyacrylamide gel containing 7 M urea in TBE (89 mM Tris, 89 mM boric acid, and 2.0 mM EDTA).[18] The reaction products were visualized by autoradiography of the gel.

Time Course of DNA Religation by the Modified Topoisomerase Analogue Containing Thiophenylalanine. The cleavage reaction mixtures (40 μL) containing 50 mM Tris-HCl (pH 7.5 for wild-type and pH 7.0 for the thiophenylalanine analogue), 40 fmol of 5′-^{32}P-labeled 18-mer/30-mer DNA substrate, and *in vitro* translated topoisomerase (20 ng of wild-type or 40 ng of modified topoisomerase) were incubated at 37 °C for 2 h. Then 12 pmol of 5′-OH 18-mer acceptor strand d(ATTCCGATAGTGACTACA) was added, and incubation was continued at 37 °C. Aliquots of the reaction were quenched with 1% SDS at 5, 10, 20, 30, and 60 s. The reaction mixture was treated with 3 volumes of ethanol and precipitated to remove SDS. Then 10 μL of 50% formamide was added to the mixture, which was heat-denatured, and then analyzed by electrophoresis on a 20% polyacrylamide gel containing 7 M urea in TBE (89 mM Tris, 89 mM boric acid, and 2.0 mM EDTA).[18] The reaction products were visualized by autoradiography of the gel.

Plasmid Relaxation Assay of Vaccinia Topoisomerase IB Analogues in the Presence of Different Metal Ions. A reaction mixture (10 μL total volume) containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgCl_2$ or $MnCl_2$, 2 mM EDTA, and 250 ng of supercoiled pSP64 plasmid DNA was treated with topoisomerase IB (0.3 ng of wild-type or 1.5 ng of modified topoisomerase) and incubated at 37 °C. Aliquots of the reaction were quenched by addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue) at 5, 10, 20, 30, 60, and 90 min. The reaction mixture was analyzed by 1% agarose gel electrophoresis.[40] After electrophoresis in 89 mM Tris buffer, pH 8.0, containing 89 mM boric acid and 2 mM EDTA at 100 V for 1 h, the gel was stained with ethidium bromide for 30 min and then rerun for 30 min to separate the relaxed (form IV) and nicked (form II) DNAs.[39] Gels were visualized using UV light, and the extent of reaction (expressed as the percentage of DNA plasmid relaxed by vaccinia topoisomerase IB) was quantified by utilizing ImageQuant version 5.2 software.

RESULTS

Synthesis of *N*-(6-Nitroveratryloxycarbonyl)-thiophenylalanyl-pdCpA *n*-Butyl Disulfide. Thiophenylalanine *n*-butyl disulfide (**1**)[32] was treated with 6-nitroveratryl chloroformate (NVOC-Cl) in 1:1 dioxane−H_2O in the presence of $NaHCO_3$[33] to afford the *N*-6-nitroveratryl derivative (**2**) in 56% yield (Scheme 2). Compound **2** was treated with chloroacetonitrile and triethylamine in acetonitrile to provide the respective cyanomethyl ester (**3**)[34] in 57% yield. Compound **3** was then treated with the tetra-*n*-butylammonium salt of pdCpA[35] in DMF. After being maintained at room temperature for 36 h, the reaction mixture was purified by C_{18} reversed phase HPLC to afford the monoacylated pdCpA derivative (**4**) in 45% yield.

Ligation of Suppressor tRNA-C_{OH} with *p*-Thiophenylalanyl-pdCpA. Suppressor $tRNA^{Phe}_{CUA}$[35] lacking the 3′-terminal cytidine and adenosine moieties was ligated to *N*-(6-nitroveratryloxycarbonyl)thiophenylalanyl-pdCpA *n*-butyl disulfide via the agency of T4 RNA ligase (Figure 1A, lane 2). As a control, *N*-(4-pentenoyl)phenylalanyl-pdCpA was also ligated to suppressor $tRNA^{Phe}_{CUA}$ (Figure 1A, lane 3). Following the ligation reaction, the *n*-butyl disulfide of *N*-(6-nitroveratryloxycarbonyl)thiophenylalanyl-$tRNA_{CUA}$ was cleaved reductively using 100 mM DTT. Irradiation of the NVOC-protected thiophenylalanyl-tRNA with a 500 W mercury−xenon lamp using both Pyrex and water filters resulted in deprotection of the 6-nitroveratryloxycarbonyl group. In the case of *N*-(4-pentenoyl)phenylalanyl-$tRNA_{CUA}$, the pentenoyl protecting group was removed using 5 mM aqueous I_2.[30,31]

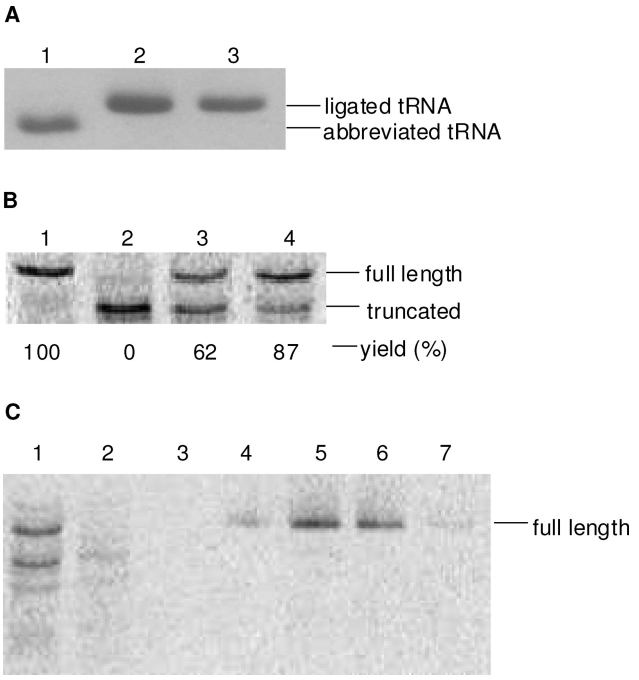

Figure 1. Preparation of vaccinia topoisomerase samples. (A) Ligation of suppressor tRNA-C_{OH} with *p*-thiophenylalanyl-pdCpA and phenylalanyl-pdCpA via the agency of T4 RNA ligase. Lane 1, abbreviated tRNA (tRNA-C_{OH}); lane 2, protected, full length *p*-thiophenylalanyl-tRNA; lane 3, protected, full length phenylalanyl-tRNA. The analysis was carried out by polyacrylamide gel electrophoresis under acidic conditions.[36] (B) SDS−PAG analysis of *in vitro* synthesis of vaccinia topoisomerase IB analogues modified at position 274. Lane 1, wild-type topoisomerase IB; lane 2, truncated topoisomerase IB (synthesized in the presence of tRNA-C_{OH} lacking any amino acid); lane 3, modified topoisomerase IB containing *p*-thiophenylalanine at position 274; lane 4, modified topoisomerase IB containing phenylalanine at position 274. The suppression efficiencies (relative to wild-type topoisomerase synthesis) are shown below each lane. (C) Purification of vaccinia topoisomerase IB analogues modified at position 274 on Ni-NTA column. Lane 1, crude sample; lane 2, flow through; lane 3, elution with 50 mM imidazole; lane 4, first elution with 150 mM imidazole; lane 5, second elution with 150 mM imidazole; lane 6, third elution with 150 mM imidazole; lane 7, fourth elution with 150 mM imidazole.

***In Vitro* Synthesis of Modified Vaccinia Topoisomerases I.** The analogues of vaccinia topoisomerase IB modified at position 274 were prepared from a DNA construct containing a TAG codon at position 274 and having a hexahistidine motif fused to the C-terminus to facilitate purification. Coupled transcription/translation in a cell-free system utilizing *E. coli* S-30 extract afforded the modified

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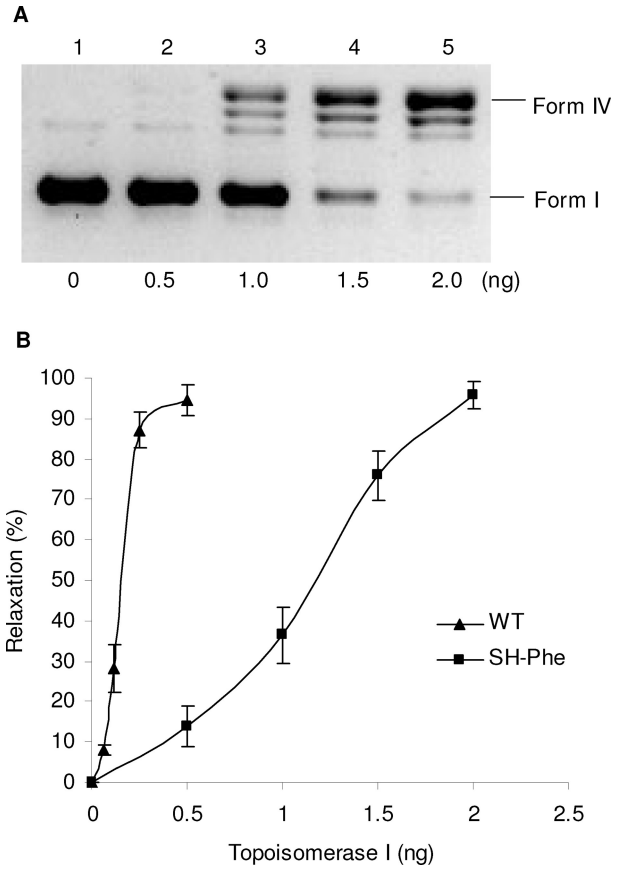

Figure 2. (A) Electrophoretic analysis of DNA relaxation mediated by four different concentrations of vaccinia topoisomerase IB containing thiophenylalanine at position 274. The relaxation assay was carried out by incubation of 0.5, 1.0, 1.5, or 2.0 ng of modified topoisomerase I with 250 ng of supercoiled plasmid pSP64 at 37 °C for 30 min. (B) Concentration dependence of DNA relaxation by wild-type topoisomerase I and modified topoisomerase I containing thiophenylalanine at position 274.

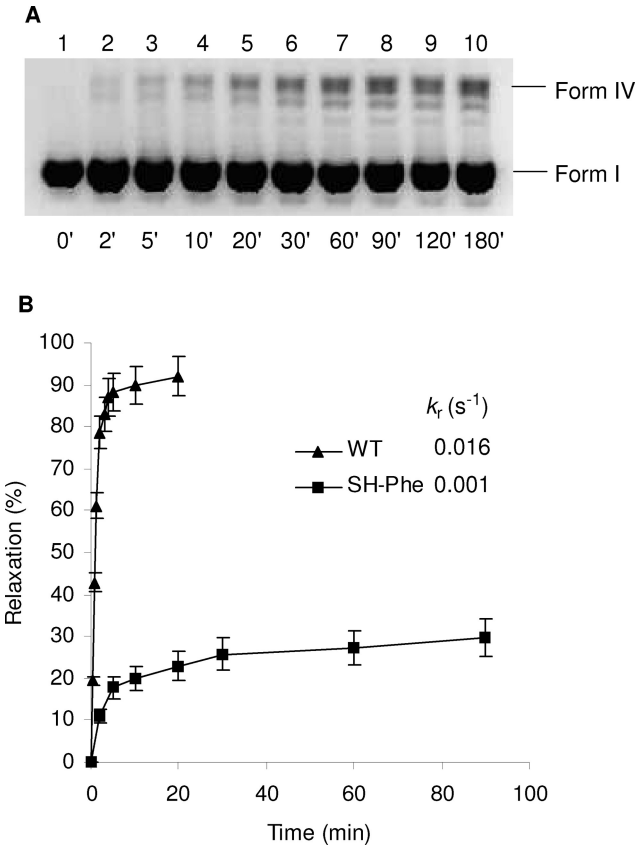

Figure 3. (A) Agarose gel electrophoretic analysis of plasmid DNA relaxation mediated by vaccinia topoisomerase IB containing thiophenylalanine at position 274. The reaction was carried out as described in Materials and Methods. Aliquots were removed at predetermined times over a period of 180 min, and the reaction was quenched by adding 1% aqueous SDS. (B) Time course of plasmid DNA relaxation comparing the wild-type enzyme and vaccinia topoisomerase IB containing thiophenylalanine. The relaxtion rate constants (k_r) were calculated by fitting the normalized data to the equation [100 − % relaxation] = $100e^{-kt}$.

proteins of interest. The topoisomerase I containing thiophenylalanine was formed with 62% suppression efficiency (Figure 1B, lane 3).[37] In comparison, the analogue containing phenylalanine at the same position was formed with a suppression efficiency of 87% (Figure 1B, lane 4). These suppression efficiencies are expressed relative to the full length wild-type topoisomerase IB synthesized by transcription and translation from a DNA template containing the wild-type vaccinia topoisomerase gene (Figure 1B, lane 1). The analogues of vaccinia topoisomerase IB were purified by Ni-NTA chromatography (Figure 1C).

Relaxation of Supercoiled pSP64 Plasmid DNA by Vaccinia Topoisomerase IB Analogues. The modified and wild-type vaccinia topoisomerases I were assayed for their ability to relax supercoiled plasmid DNA. Relaxation activity was determined by incubation of 1 ng of the purified enzymes with 250 ng of supercoiled plasmid DNA in a 10 μL reaction mixture at 37 °C for 30 min. The reactions were analyzed by running a 1% agarose gel sequentially in the absence and then in the presence of ethidium bromide to separate the relaxed plasmid DNA (form IV) from the supercoiled plasmid DNA (form I) and (adventitious) nicked plasmid DNA (form II). The modified topoisomerase IB containing thiophenylalanine at position 274 exhibited some DNA relaxation activity (Figure S1, lane 3), albeit less than that of wild-type topoisomerase IB (Figure S1, lane 2). As anticipated, the topoisomerase analogue containing phenylalanine at position 274 lacked any topoisomerase activity (Figure S1, lane 4).

Concentration Dependence of DNA Relaxation Assay. The dose dependence of DNA relaxation by wild-type topoisomerase I and the modified topoisomerase I containing thiophenylalanine at position 274 were compared as shown in Figure 2. The specific activity of the wild-type enzyme was found to be greater than that of the modified topoisomerase I. However, the modified topoisomerase could attain similar levels of relaxation as wild-type topoisomerase, when 2 ng of modified topoisomerase was used in a 10 μL reaction mixture and was incubated at 37 °C for 30 min.

Kinetics of DNA Relaxation by Vaccinia Topoisomerases IB. The time course of DNA relaxation is shown in Figure 3, and the rate constants (k_r) were calculated by fitting the normalized data to the equation [100 − % relaxation] = $100e^{-kt}$. The rate of DNA relaxation of wild-type vaccinia topoisomerase IB was about 16 times greater than that of the modified topoisomerase IB containing thiophenylalanine at position 274.

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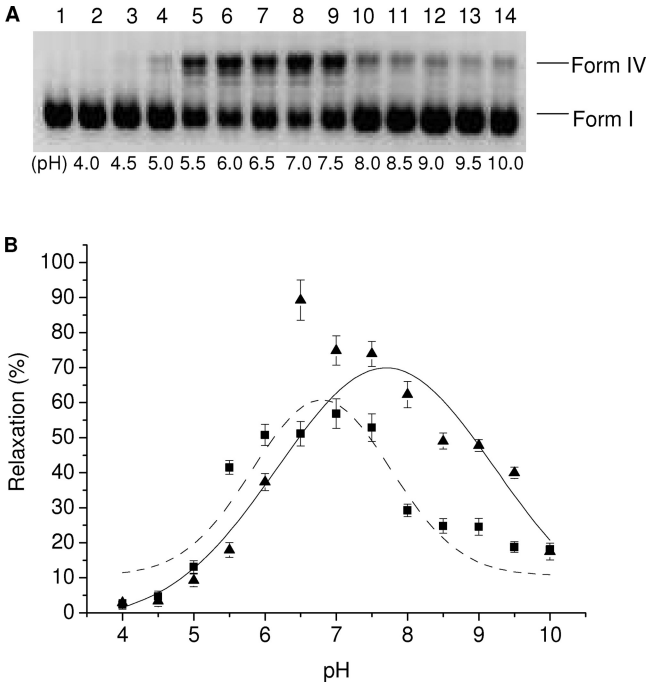

Figure 4. (A) Agarose gel electrophoretic analysis of the pH dependence of DNA relaxation mediated by vaccinia topoisomerase IB containing thiophenylalanine. Lane 1, pSP64 plasmid DNA alone; lanes 2−14, plasmid DNA relaxation assay at different pH values, as indicated. (B) pH profile of plasmid DNA relaxation comparing the activity of (1 ng of) wild-type topoisomerase IB (▲) and (1.5 ng of) vaccinia topoisomerase IB containing thiophenylalanine (■).

pH Dependence of Topoisomerase-Mediated DNA Relaxation. The relaxation activity of the modified topoisomerase IB analogue was investigated over a range of pH values and compared with that of the wild-type topoisomerase IB. The supercoiled plasmid DNA (form I) was treated with one of the topoisomerases IB and incubated at 37 °C at each of several pH values (pH 4.0−10.0). As shown in Figure 4, the pH dependence of DNA relaxation gave a bell-shaped curve for each of the enzymes. However, the pH maximum for the modified topoisomerase IB analogue containing thiophenylalanine was shifted to lower pH by about 1 pH unit when compared to wild-type topoisomerase IB. The modified topoisomerase IB analogue was found to function well, albeit in a narrower pH range (pH 5.5−7.5) compared to the wild-type topoisomerase (pH 6.0−9.5). These results presumably reflect the lower pK_a of SH (pK_a ≈ 6.6) as compared with that of OH (pK_a ≈ 9.9) groups[41] at position 274 of vaccinia topoisomerase IB as well as other factors.

DNA Single-Turnover Cleavage by the Modified Topoisomerase I Containing Thiophenylalanine. Single-turnover cleavage assays were carried out using lower DNA concentrations to determine the effects of the thiophenylalanine modification on the rate of the cleavage step.[18] A suicide DNA substrate containing a single CCCTT cleavage site was used to examine the cleavage transesterification reaction under single-turnover conditions. The substrate (shown in Figure 5A) consisted of a 5′-^{32}P-labeled 18-mer scissile strand 5′-pCGTGTCGCCCTTATTCCC annealed to an unlabeled 30-mer strand. The cleavage reaction of *in vitro* translated vaccinia topoisomerase analogues resulted in covalent attachment of the ^{32}P-labeled 12-mer 5′-pCGTGTCGCCCTTp to the enzymes.

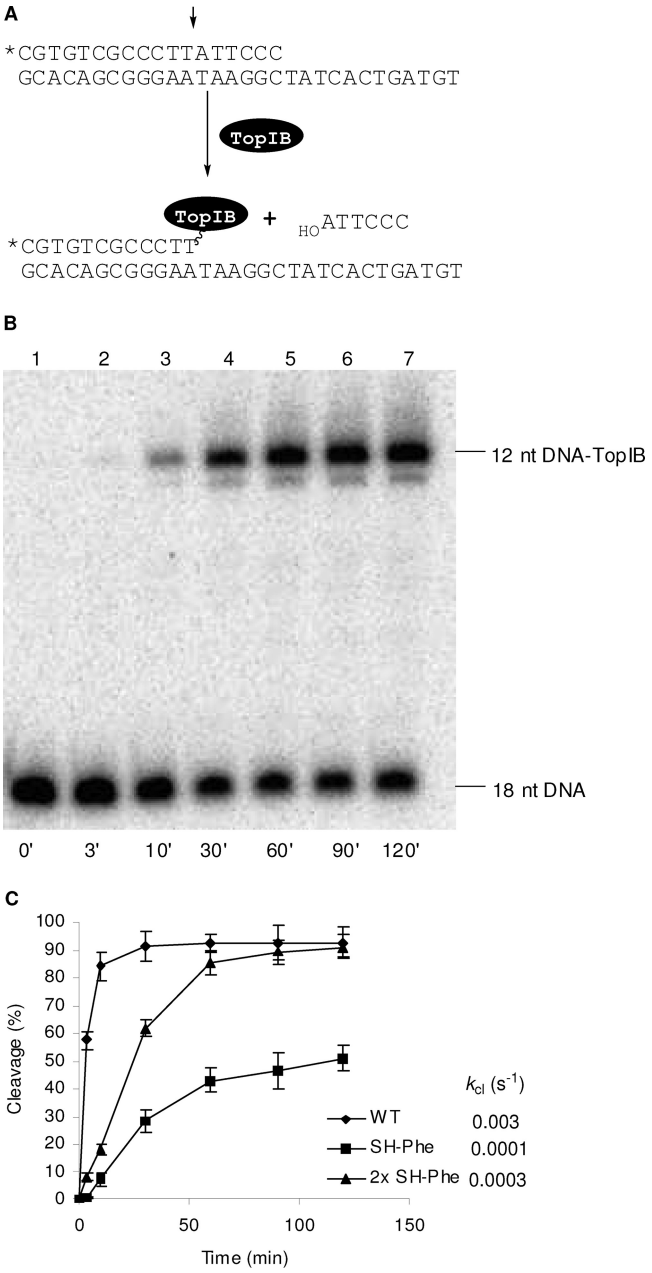

Figure 5. (A) Illustration of single-turnover DNA cleavage mediated by vaccinia topoisomerase IBs. (B) Electrophoretic analysis on a PAG containing 7 M urea of single-turnover DNA cleavage mediated by vaccinia topoisomerase IB containing thiophenylalanine. The reaction was carried out as described in Materials and Methods. Aliquots were removed at predetermined times over a period of 120 min, and the reaction was quenched by adding 1% aqueous SDS. (C) Time course of DNA cleavage comparing the wild-type (20 ng) and thiophenylalanine-containing (20 and 40 ng) vaccinia topoisomerases IB. The cleavage rate constants (k_{cl}) were calculated by fitting the normalized data to the equation [100 − % cleavage] = 100e^{-kt}.

The reaction was effectively irreversible because the cleaved 3′-nucleotide (ATTCCC) would not be able to maintain a stable duplex structure and so would dissociate from the noncleaved strand. At the low concentration at which this hexanucleotide was present, reassociation with the nonscissile strand was unlikely. It was found that the site of partial duplex cleavage was unchanged by the presence of thiophenylalanine at position 274, but the rate of cleavage diminished significantly (Figure

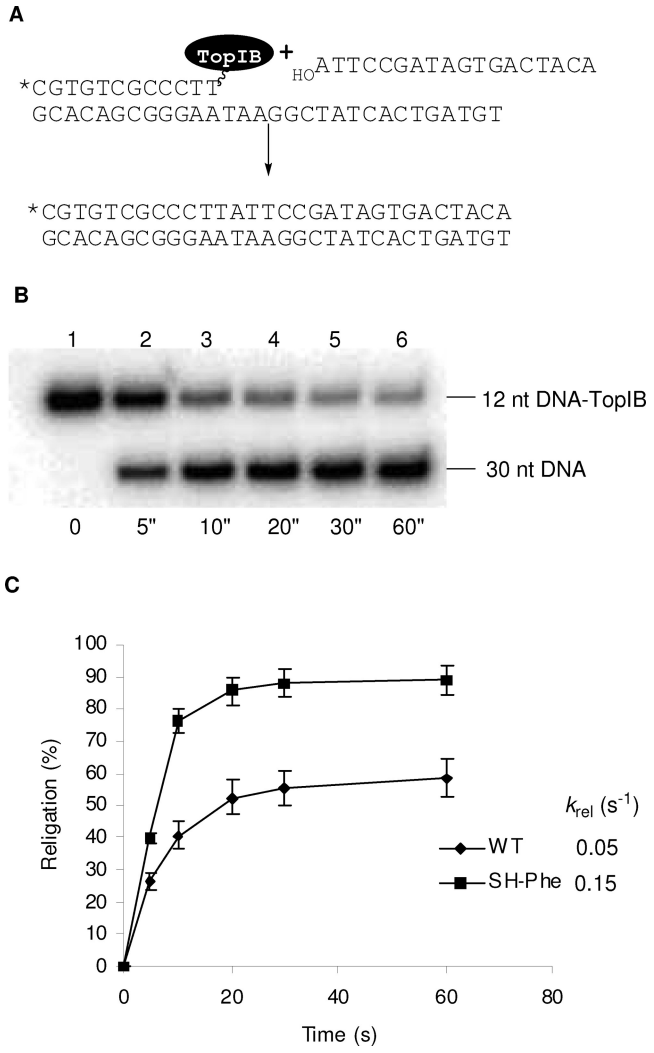

Figure 6. (A) Illustration of single-turnover DNA religation mediated by vaccinia topoisomerase IBs. (B) Electrophoretic analysis on a PAG containing 7 M urea of single-turnover DNA religation mediated by modified vaccinia topoisomerase IB containing thiophenylalanine. The reaction was carried out as described in Materials and Methods. Aliquots were removed at predetermined times over a period of 60 s, and the reaction was quenched by adding 1% aqueous SDS. (C) Time course of religation comparing the wild-type and modified vaccinia topoisomerases IB. The religation rate constants (k_{rel}) were calculated by fitting the normalized data to the equation [100 − % religation] = $100e^{-kt}$.

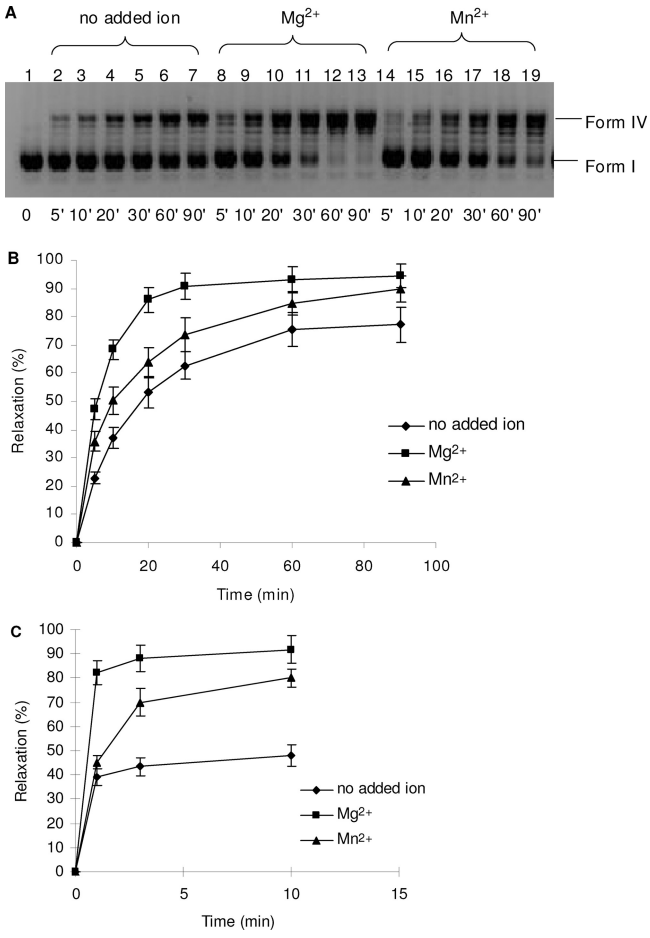

Figure 7. (A) Agarose gel electrophoretic analysis of DNA relaxation mediated by modified vaccinia topoisomerase IB containing thiophenylalanine in the presence of different metal ions. The reaction was carried out as described in Materials and Methods. Aliquots were removed at predetermined times over a period of 90 min, and the reaction was quenched by adding 1% aqueous SDS. (B) Time course of relaxation mediated by modified vaccinia topoisomerase IB containing thiophenylalanine in the presence of different metal ions. (C) Time course of relaxation mediated by wild-type vaccinia topoisomerase IB comparing different ions.

5B,C). The DNA cleavage rate for the modified topoisomerase IB was 30 times lower than that obtained by same amount of wild-type topoisomerase IB (Figure 5C). Although 20 ng of the modified enzyme did not effect complete cleavage of the DNA substrate, complete cleavage was obtained when 40 ng of the modified enzyme was employed (Figure 5C).

Rate of DNA Religation by the Modified Topoisomerase IB Analogue Containing Thiophenylalanine. The effect of the tyrosine modification on the religation reaction was also studied under single-turnover conditions by assaying the ability of preformed suicide intermediates to transfer the covalently bound 5′-^{32}P-labeled 12-mer strand to a 5′-OH terminated complementary 18-mer strand, producing a 30-mer DNA duplex product (Figure 6A). After forming the suicide intermediate on the 12-mer/30-mer DNA substrate,[16,18] the religation reaction was initiated by adding a 300-fold molar excess of the 18-mer DNA acceptor, which was fully complementary to the 5′ single-stranded region of the suicide intermediate. Aliquots were withdrawn immediately prior to the addition of 18-mer (defined as time 0) and at several times afterward. The religation reaction carried out using the modified topoisomerase prepared by translation revealed a time-dependent depletion of the covalent adduct and concomitant accumulation of the 30-mer duplex strand transfer product (Figure 6B). The extent of religation at each time point was quantified as the fraction of the ^{32}P-labeled DNA present as covalent adduct at time 0 that was converted to 30-mer strand transfer product. As shown in Figure 6C, religation of the covalent intermediate containing the modified topisomerase IB was 3-fold faster than that of the wild-type enzyme.

Relaxation of Supercoiled pSP64 Plasmid DNA by Topoisomerases IB in the Presence of Different Metal Ions. The wild-type and modified topoisomerases were assayed for their ability to relax supercoiled plasmid DNA in the presence of the divalent metal ions Mg^{2+} and Mn^{2+}. As

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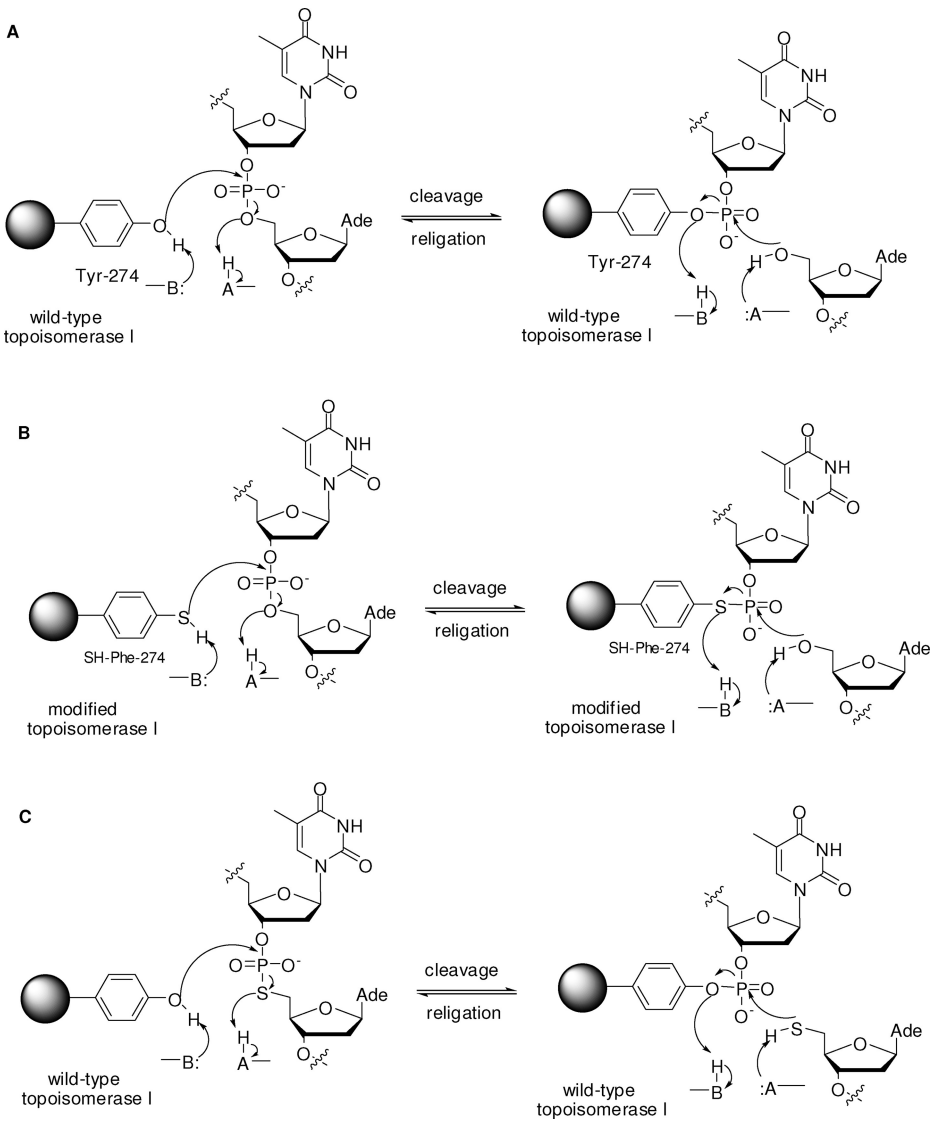

Figure 8. Mechanism of DNA relaxation by wild-type and modified DNA topoisomerases IB.

shown in Figure 7, both divalent metal ions increased the rate of DNA relaxation (Figure 7A). Interestingly, the wild-type enzyme exhibited larger percentage increases in the rate of relaxation in the presence of added Mg^{2+} and Mn^{2+} (Figure 7B,C). DNA relaxation by the modified topoisomerase I was also facilitated. In both cases, the relaxation rates realized by admixture of 8 mM $MgCl_2$ were greater than those achieved with the same concentration of $MnCl_2$.

■ DISCUSSION

Topoisomerase I-mediated DNA relaxation is a multistep process, which includes noncovalent binding between the enzyme and its DNA duplex substrate, sequence-selective cleavage, strand passage, religation, and dissociation.[2,3] Alteration of any of these steps can result in a change in efficiency of the overall relaxation process. To facilitate a better understanding of the multistep process of topoisomerase I-mediated DNA relaxation, the effects of replacing the phenolic active site tyrosine OH group with an SH group have been studied. The parameters measured include the relative rates of (multiturnover) DNA relaxation by the wild-type and modified topoisomerases as well as single turnover DNA cleavage and religation reactions.

A key feature of the suicide cleavage reaction is that the unlabeled 6-mer strand ATTCCC produced by the cleavage event dissociates spontaneously from the protein−DNA complex. Loss of this strand effectively drives the reaction to completion so that the reaction can be treated kinetically as a first-order unidirectional process. From the polyacrylamide gel (Figure 5B), both the tyrosine-274 and thiophenylalanine-274 mediated covalent protein−DNA complexes could be observed. Since the enzyme containing Phe274 was found to be inactive, this suggested that the modified topoisomerase IB having thiophenylalanine at position 274 mediated DNA relaxation using the same basic mechanism as the wild-type topoisomerase IB (Figure 8A,B). The rate of substrate DNA cleavage by the modified topoisomerase was 30 times lower than that of the wild-type enzyme (Figure 5C). In comparison, the rate of DNA religation mediated by the modified enzyme was 3-fold faster (Figure 6C). It may be noted that this is in the same order as the P−S and P−O bond energies; theoretical and experimental measurements suggest that the P−S bond is significantly weaker than the P−O bond, although both are influenced by

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the other atoms bound to P.[42−44] Thus, the more facile breakage of the P−S bond in the modified topoisomerase I−DNA oligonucleotide binary complex concomitant with DNA religation (Figure 6C) is consistent with expectations based on energetic considerations. Conversely, the formation of the covalent binary complex with the modified topoisomerase I (establishing a P−S bond at the expense of a P−O bond) is slower than DNA cleavage by the wild-type enzyme (which involves the formation and loss of P−O bonds) (Figure 8A).

The reversible DNA cleavage reactions mediated by the wild-type and modified topoisomerases I are summarized in parts A and B of Figure 8, respectively.[45,46] The data are consistent with operation of the same basic mechanisms in both cases, albeit with differences in the energy profiles for DNA cleavage and religation based on the relative P−O and P−S bond strengths of the formed enzyme−DNA covalent intermediates. In this context, it is interesting to consider earlier studies involving eukaryotic topoisomerases I.

Burgin et al. studied the religation of DNA topoisomerase I using an oligonucleotide substrate with a bridging P−S at the scissile bond.[47] Incubation of this S-containing substrate with DNA topoisomerase I resulted in facile cleavage that was reported to be irreversible. This observation was later employed to produce a covalent topoisomerase I−DNA covalent binary complex that was crystallized in support of X-ray crystallographic analysis of topoisomerase I−DNA structure determination.[48]

However, while cleavage of the bridging P−S bond at the preferred DNA cleavage sequence was clearly a favorable process (Figure 8C), Henningfeld et al.[16] and Krogh et al.[49] later employed a 5′-SH substrate in a religation experiment analogous to that in Figure 6A to demonstrate unequivocally that religation of the DNA (with (re)formation of a P−S bond) was entirely feasible (Figure 8C). In these experiments, the cleavage−religation equilibrium favored the cleavage reaction, again consistent with the relative strengths of the P−S and P−O bonds broken and formed in the two processes.

In this present study, a cleavage−religation equilibrium was found again. Since the thiol group was present in the enzyme, rather than in the DNA substrate, the equilibrium favored religation. This provides further evidence that cleavage of S-bridged oligonucleotides by DNA topoisomerase I is a reversible process.

Previous studies have shown that vaccinia topoisomerase IB has no metal ion requirement[3,40] but that the addition of divalent metal ions such as Mg^{2+} and Mn^{2+} could increase the activity of wild-type vaccinia topoisomerase IB.[40] To determine how divalent metal ions affected the modified vaccinia topoisomerase containing thiophenylalanine, DNA relaxation assays were carried out in the presence of additional Mg^{2+} and Mn^{2+} (Figure 7). Mg^{2+} and Mn^{2+} both increased the activity of the modified vaccinia topoisomerase IB (Figure 7B). In parallel with observations made for wild-type topoisomerase, the modified vaccinia topoisomerase exhibited the best activity in the presence of Mg^{2+} (Figure 7B,C). Typically, if a wild-type enzyme uses Mg−O interaction for catalysis, replacement of Mg^{2+} by Mn^{2+} diminishes enzyme function.[50] Enzymes utilizing metal ion−S interaction exhibit the reverse effect since thiol group interaction with Mn^{2+} is favored.[51] In this study, the fact that both metals have the same effects on wild-type and thiophenylalanine-modified vaccinia topoisomerase I argues that no metal ion−heteroatom interaction is required for catalysis. It seems likely that the divalent metal ions increase the activity of vaccinia topoisomerase I by binding to the DNA substrates and altering their secondary structure or facilitating enzyme−DNA binding. Additional experiments are required to define the nature of the metal ion effects more exactly.

In the aggregate, the foregoing data support the interpretation that the wild-type and modified vaccinia topoisomerases IB effect DNA relaxation by the same mechanism (Figure 8A,B).[45,46] The SH group of thiophenylalanine at position 274 of the modified enzyme mediates a nucleophilic attack on phosphodiester backbone of DNA to form a DNA-(3′-*S*-phosphotyrosyl)-enzyme intermediate, with the formation of a free DNA strand having a 5′-OH group (Figure 8B). The free DNA strand can undergo passage around the unbroken strand, removing DNA supercoils; attack of the DNA 5′-OH group upon the covalent enzyme−DNA intermediate then regenerates the original strand and free topoisomerase IB. During this process, the cleavage step is the rate-limiting step for the modified enzyme.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

DNA plasmid relaxation by vaccinia topoisomerase IB analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; IPTG, isopropyl β-D-thiogalactopyranoside; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; NVOC, nitroveratryloxycarbonyl.

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