Effects of Modification of the Active Site Tyrosine of Human DNA Topoisomerase I†

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ABSTRACT: The human topoisomerase I-mediated DNA relaxation reaction was studied following modification of the enzyme at the active site tyrosine (position 723). A series of unnatural tyrosine analogues was incorporated into the active site of human topoisomerase I by utilizing misacylated suppressor tRNAs in an in vitro protein synthesizing system. The relaxation activities of the modified human topoisomerase I analogues having varied steric, electronic, and stereochemical features were all greatly diminished relative to that of the wild type. It was found that modifications involving replacement of the nucleophilic tyrosine OH group with NH_2, SH, or I groups eliminated DNA relaxation activity, as did changing the orientation of the nucleophilic tyrosine OH group. Only tyrosine analogues having the phenolic OH group in the normal position with respect to the protein backbone were active; the relative activities could be rationalized in chemical terms on the basis of the H-bonding and the electronic effects of the substituents attached to the meta position of the aromatic ring. In addition, the poisoning of one of the modified human topoisomerase I analogues, as part of covalent binary complexes with DNA, by CPT and 20-thio CPT was evaluated.

Human topoisomerase I, a 97 kDa monomeric protein, has been identified as a key enzyme in the regulation of DNA topology; the enzyme functions by mediating transient single-strand breakage of the DNA substrate to release the supercoiling stress of the DNA duplex. As such, this enzyme plays an important role in essential nuclear processes such as DNA replication, transcription, and recombination (*1*−*7*). In recent years, interest in the study of human topoisomerase I has expanded into the realms of pharmacology and medicine, since this enzyme has been identified as a specific target of the anticancer agent camptothecin (CPT)[1] (*8*−*10*).

The basic mechanism of DNA relaxation by human topoisomerase I involves a reversible transesterification reaction, as illustrated in Figure 1. The phenolic OH group of the active site tyrosine residue (723) attacks the phosphate ester in the backbone, forming a DNA−(3′-*O*-phosphotyrosyl)−topoisomerase I covalent complex with a free DNA strand having a 5′-OH group. After DNA supercoiling has been removed as a result of the free DNA strand being passed around the unbroken DNA strand, the original DNA duplex can be regenerated by attack of the free DNA 5′-OH group upon the covalent enzyme−DNA complex, with concomitant release of free topoisomerase I (*11*−*13*).

Further investigation of the topoisomerase I-mediated transesterification reaction has been carried out previously using modified DNA substrates (*14*−*20*). One recent study of vaccinia topoisomerase I focused on the mechanism of the transesterification reaction by topoisomerases modified at the active site tyrosine residue. The nucleophilic OH group was found to tolerate relatively few changes in the active site residue (*21*). Herein, we describe an analogous study involving Tyr723 of human topoisomerase I. This study employed 13 structural analogues of tyrosine (Figure 2). In addition, the ability of the topoisomerase I poisons camptothecin (CPT) and 20-thio CPT to inhibit a modified human topoisomerase I was evaluated.

EXPERIMENTAL PROCEDURES

General Methods and Materials. The pdCpA derivatives of tyrosine analogues were prepared as described previously (*21*−*23*). Plasmid pGEM-hTop1(wt) containing the human topoisomerase I gene was obtained from M.-A. Bjornsti. Endonucleases *Bam*HI, *Eco*RI, *Fok*I, and *Dpn*I as well as *Taq* DNA ligase, T4 DNA ligase, T4 polynucleotide kinase, and T4 RNA ligase were purchased from New England Biolabs (Beverly, MA). *Pfu*I DNA polymerase was purchased from Stratagene Cloning Systems (La Jolla, CA). The DNA primers for mutagenesis were ordered from Integrated DNA Technologies (Coralville, IA). BL21(DE3)pLysS and JM109 *Escherichia coli* competent cells, TNT quick coupled transcription/translation system, and the Wizard plus DNA purification system were purchased from Promega Corp. (Madison, WI). [^{35}S]Methionine (1000 Ci/mmol, 10 μCi/μL) and heparin agarose were obtained from Amersham Corp. (Piscataway, NJ). AmpliScribe transcription kits were obtained from Epicentre Technologies (Madison, WI). Ultra-

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[1] Abbreviations: BSA, bovine serum albumin; CPT, camptothecin; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; DEPC, diethyl pyrocarbonate; EtOH, ethanol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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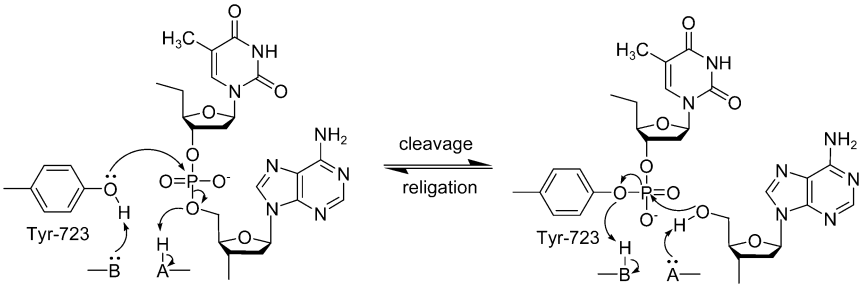

FIGURE 1: Human topoisomerase I-mediated transesterification reaction, leading to DNA relaxation.

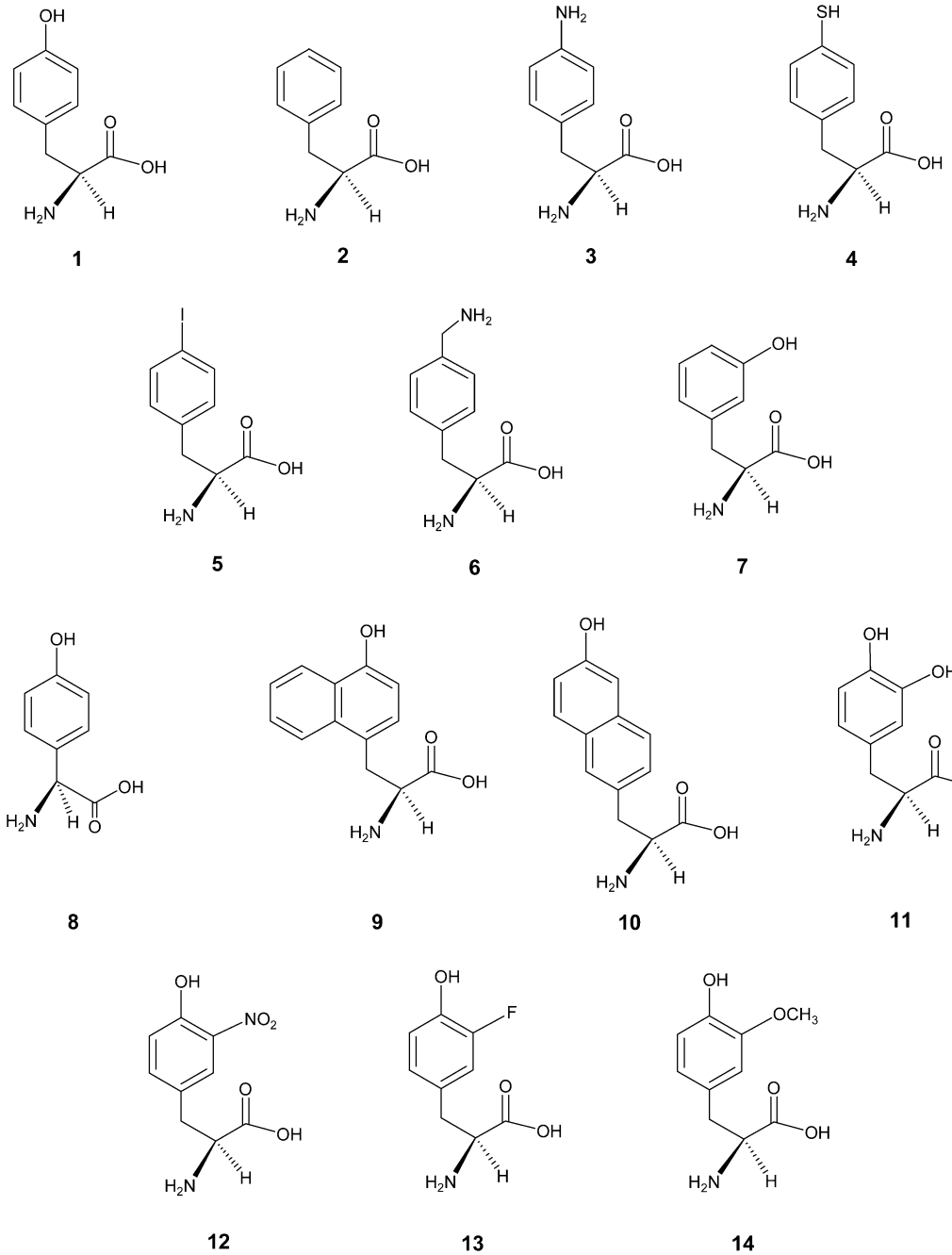

FIGURE 2: Tyrosine analogues incorporated into human topoisomerase I at position 723. Analogues **7**, **9**, **10**, and **13** were prepared in racemic form.

pure agarose was from Bethesda Research Laboratories (Bethesda, MD).

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 apparatus equipped with ImageQuant version 5.2 from Molecular Dynamics. UV spectral measurements were taken using a Perkin-Elmer Lambda 20 UV−vis spectrometer.

General Procedure for the Synthesis of pdCpA Derivatives of Tyrosine Analogues. To a conical vial containing 5 μmol of protected amino acid cyanomethyl ester was added a

solution containing 1 μmol of the tris(tetrabutylammonium) salt of pdCpA in 50 μL of anhydrous DMF, followed by 10 μL of Et_3N. The reaction mixture was kept in the dark and stirred at room temperature for 36 h, and the progress of the reaction was monitored by HPLC. Monitoring involved 5 μL aliquots of the reaction mixture, which were diluted with 45 μL of a 1:1 CH_3CN/50 mM NH_4OAc buffer (pH 4.5). Ten microliters of the diluted aliquot was analyzed by HPLC on a C_{18} reversed-phase column (250 mm $\times$ 10 mm). The column was washed with a linear gradient of 1 $\rightarrow$ 63% CH_3CN in 50 mM NH_4OAc buffer (pH 4.5) over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). After 3 days, the reaction mixture was diluted to a total volume of 600 μL of 1:1 CH_3CN/50 mM NH_4OAc buffer (pH 4.5) and purified on a semipreparative C_{18} reversed-phase HPLC column. After lyophilization of the appropriate fractions, the product was obtained as a solid. The characterization of individual tyrosyl-pdCpA derivatives has been reported previously (*21*, *23*).

Site-Directed Mutagenesis of Position 723 of Human Topoisomerase I (24). The primer for modification of Tyr723 was GA ACC TCC AAA CTC AAT TAG CTG GAC CCT AGG ATC AC. This primer (200 pmol) was phosphorylated at the 5′-end with T4 polynucleotide kinase (10 units).

A polymerase chain reaction was carried out in a 50 μL (total volume) reaction mixture containing 300 ng of template plasmid DNA, 24 pmol of primer, 25 nmol of dNTPs, 5 units of cloned *Pfu* DNA polymerase, and 40 units of *Taq* DNA ligase in 20 mM Tris-HCl (pH 8.2), 12.5 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM KCl, 5 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 5 mM DTT, 0.5 mM NAD, 0.1% Triton X-100, and 0.05 mg/mL BSA. The thermal cycle was performed as follows: preincubation at 65 °C for 5 min to allow the ligase to repair any nicks in the template; initial denaturation at 95 °C for 2 min; 18 cycles at 95 °C for 1 min, 45 °C for 1 min, and 65 °C for 20 min; and postincubation at 75 °C for 7 min. To remove the methylated and hemimethylated wild-type DNA template, the restriction enzyme *Dpn*I (20 units) was added to the reaction mixture which was incubated at 37 °C for 1 h. The reaction product was then denatured at 95 °C for 1 min, followed by two cycles at 95 °C for 1 min, 51 °C for 1 min, and 70 °C for 24 min. After ethanol precipitation, the reaction product was redissolved in 10 μL of H_2O.

Five microliters of the final sample was transformed into 50 μL of BL21(DE3)pLysS *E. coli* competent cells (transformation efficiency of $\sim 10^7$ cfu/μg), plated on LB agar containing ampicillin and chloramphenicol, and incubated overnight at 37 °C. The colonies were subcoloned on a fresh LB agar plate supplied with ampicillin and IPTG. Plasmids were isolated from the new colonies and screened by restriction analysis and in vitro translation in TNT rabbit reticulocyte lysate. The sequence of the modified human topoisomerase I gene containing an amber stop codon, TAG, at the position corresponding to Tyr723 was confirmed by DNA sequence analysis.

In Vitro Transcription of Abbreviated Suppressor tRNA-C_{OH} (25). Plasmid pYRNA8, encoding the yeast suppressor $tRNA^{Phe}_{CUA}$, was linearized with *Fok*I, followed by transcription using an AmpliScribe T7 RNA polymerase transcription kit. A reaction mixture (300 μL total volume) containing 20 mM Tris-acetate (pH 7.9), 10 mM $Mg(OAc)_2$, 50 mM KOAc, and 120 μg of plasmid DNA was digested with 120 units of *Fok*I. The reaction mixture was incubated at 37 °C for 4 h, extracted with a phenol/chloroform mixture, and precipitated by treatment with 3 volumes of cold ethanol.

The transcription reaction was carried out in a buffered reaction mixture (800 μL total volume) containing ATP, CTP, and UTP (7.5 mM each), 5 mM GTP, 20 mM GMP, 10 mM DTT, 50 μg of linearized DNA template, and 80 μL of the T7 RNA polymerase preparation at 42 °C for 12 h. The elaborated tRNA-C_{OH} transcript was diluted 3-fold using 0.1 M NaOAc (pH 5.2) and applied to a 400 μL column of DEAE-Sepharose CL-6B. The column was washed with 600 μL of 0.1 M NaOAc (pH 5.2) and then eluted successively with 600 μL portions of 0.1 M NaOAc (pH 5.2) containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 0.9 M NaCl. Each of the 600 μL eluates was precipitated with 900 μL of 2-propanol for 20 min at 0 °C, washed with 70% ethanol, redissolved in 50 μL of RNase-free water, and analyzed by 8% denaturing polyacrylamide gel electrophoresis (50 V, 3 h). The fractions (0.6−0.8 M NaCl) containing $tRNA_{CUA}$-C_{OH} were pooled and stored at −80 °C.

Chemical Misacylation of Suppressor tRNA-C_{OH}. Suppressor tRNA aminoacylation was carried out in 100 μL (total volume) of 100 mM Na Hepes (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 (*22*, *23*, *26*) (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 0.1 volume of 3 M NaOAc (pH 5.3), and the aminoacylated tRNA was precipitated with 3 volumes of cold EtOH. The ligation efficiency was estimated by gel electrophoresis (pH 5.0) (*27*).

Deprotection of pentenoyl-protected aminoacyl-tRNAs was accomplished by treatment with 5 mM aqueous I_2 (30 min at 25 °C) (*21*−*23*). Following deprotection, the solution was centrifuged, and the cleared supernatant was adjusted to 0.3 M NaOAc and treated with 4 volumes of cold EtOH to precipitate the aminoacylated tRNA. The tRNA pellet was washed with 70% aqueous EtOH and then dissolved in an aqueous solution for use in in vitro suppression experiments.

NVOC-protected aminoacyl-tRNAs were deprotected at a tRNA concentration of 3 μg/μL. The aminoacyl-tRNAs were cooled to 2 °C and irradiated with a 500 W mercury−xenon lamp using both Pyrex and water filters. Typically, NVOC-protected aminoacyl-tRNAs were deblocked for 5 min. After irradiation, deblocked aminoacylated suppressor tRNAs were used in in vitro suppression experiments.

In Vitro Synthesis of Human Topoisomerase I Analogues. Human topoisomerase I and its analogues were synthesized in rabbit reticulocyte lysate using TNT quick coupled transcription/translation systems. The reaction mixture (300 μL total volume), containing 7.5 μg of DNA template and 30 μg of deprotected misacylated $tRNA_{CUA}$, was incubated at 30 °C for 2 h. As a control, in vitro translation was also carried out in the absence of misacylated $tRNA_{CUA}$. Aliquots were removed for electrophoretic analysis using 8% SDS−PAGE (*28*). Autoradiography of the gel was carried out to determine the location of the ^{35}S-labeled protein; quantification of the bands was carried out using a phosphorimager.

Protein Purification by Heparin Agarose Chromatography. Human topoisomerase I and its analogues were purified using heparin agarose chromatography modified from a published method (*29*). Following in vitro translation, the reaction

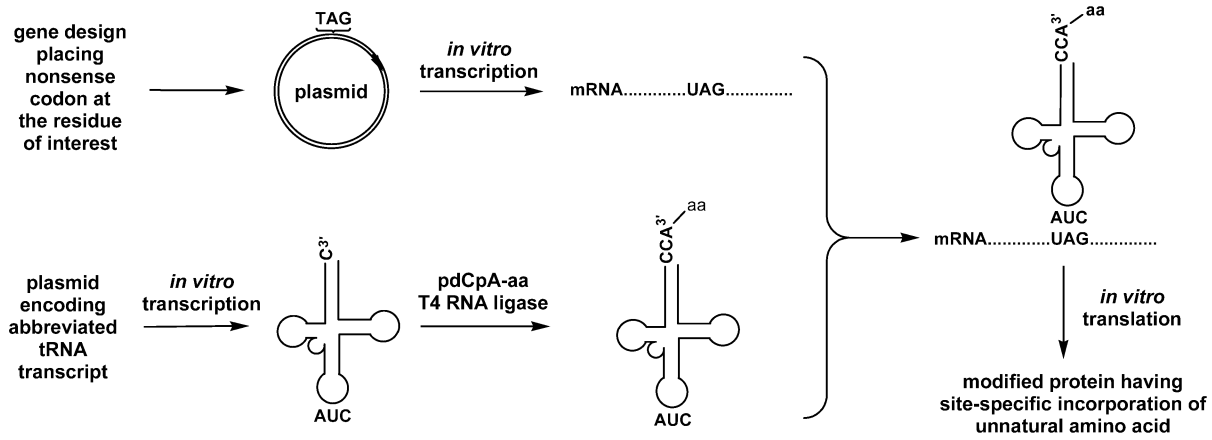

FIGURE 3: General strategy employed for elaboration of human topoisomerase I analogues modified at position 723 by incorporation of unnatural amino acids.

mixture (300 μL) was diluted with 700 μL of 10 mM Tris-HCl (pH 7.5) containing 0 M NaCl, 0.5 mM DTT, 0.5 mM EDTA and 10% glycerol and mixed gently with 50 μL of a 50% slurry of heparin agarose for 2 h at 4 °C. After centrifugation for 10 s at 14000*g*, the resin pellet was washed three times with 300 μL portions of 10 mM Tris-HCl (pH 7.5) containing 0.25 M NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 10% glycerol and once with 100 μL of 10 mM Tris-HCl (pH 7.5) containing 0.6 M NaCl, 0.5 mM DTT, 0.5 mM EDTA, 100 μg/mL BSA, and 10% glycerol. The protein was washed three times with 20 μL of 10 mM Tris-HCl (pH 7.5) containing 0.9 M NaCl, 0.5 mM DTT, 0.5 mM EDTA, 100 μg/mL BSA, and 10% glycerol. Aliquots of each fraction were analyzed by 8% SDS−PAGE. The amount of ^{35}S-labeled protein in each fraction was determined by liquid scintillation counting of a portion of each.

Plasmid Relaxation Assay. The human topoisomerase I-mediated DNA relaxation reaction was carried out in a 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 DNA plasmid, and a (modified) topoisomerase I. After incubation at 37 °C for 4 h, the reaction was quenched by addition of 3 μL of gel loading solution (2.5% SDS, 30% glycerol, and 0.125% bromophenol blue), followed by electrophoretic analysis using a 1% agarose gel. After electrophoresis in 40 mM Tris-acetate (pH 8.0) containing 2 mM EDTA at 70 V for 2 h, the gel was stained with ethidium bromide and then rerun for 1 h to separate the relaxed DNA (form IV) from nicked DNA (form II) (*30*). Gels were visualized using UV light, and the extent of reaction (expressed as the percentage of DNA plasmid relaxed by human topoisomerase I) was quantified by densitometry utilizing ImageQuant version 5.2.

Kinetics of DNA Relaxation by Human Topoisomerase I. Supercoiled DNA plasmid (250 ng) was treated with 0.04 ng of (modified) human topoisomerase I in a 10 μL reaction mixture containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2.5 mM EDTA. Each reaction mixture was incubated at 37 °C and each reaction was quenched at a predetermined time 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 in 40 mM Tris-acetate (pH 8.0) containing 2 mM EDTA at 70 V for 2 h. Following electrophoresis, the gel was stained with ethidium bromide and then rerun for 1 h to separate the relaxed DNA (form IV) from nicked DNA (form II) (*30*). Gels were visualized using UV light, and the extent of reaction (expressed as the percentage of DNA plasmid relaxed by human topoisomerase I) was quantified by densitometry utilizing ImageQuant version 5.2. The observed rate constants (k_{obs}) were determined by fitting the equation 100−% reaction extent = 100 exp(−kt).

RESULTS

In Vitro Elaboration of Human Topoisomerase I. The general strategy used for modification of human topoisomerase I at the active site tyrosine is outlined in Figure 3, as developed and employed previously for a number of other proteins (*21*, *31*−*33*). The DNA plasmid pGEM-hTop1(wt), encoding the gene for human topoisomerase I, was modified to include a nonsense (TAG) codon at active site position 723 using site-directed mutagenesis techniques to construct plasmid pGEM-hTop1(723). In parallel, suppressor $tRNA^{Phe}_{CUA}$ lacking the 3′-terminal cytidine and adenosine moieties was prepared by in vitro transcription using T7 RNA polymerase as described previously (*26*). The abbreviated $tRNA_{CUA}$-C_{OH} was coupled with N-protected 2′(3′)-*O*-aminoacyl-pdCpA derivatives via the agency of T4 RNA ligase (Figure 4). Human topoisomerase I was modified at the active site by incorporation of a number of tyrosine analogues (Figure 2) into position 723. These topoisomerase I analogues were prepared by translation in a cell-free system utilizing rabbit reticulocyte lysate. Figure 5 shows the SDS−PAGE analysis of the elaborated human topoisomerase I analogues incorporating 14 different tyrosine derivatives (**1**−**14**) into position 723. As a control, full-length protein was synthesized by transcription and translation from a DNA template containing the wild-type human topoisomerase I gene (Figure 5, lane 1). The synthesis of modified full-length human topoisomerase I by translation of the mRNA containing the UAG codon at position 723 was dependent on the presence of an aminoacylated suppressor $tRNA_{CUA}$. In the absence of the misacylated $tRNA_{CUA}$, protein translation was terminated at the nonsense codon by release factors and yielded a protein truncated at position 722 (Figure 5, lane 2). In the presence of aminoacylated $tRNA_{CUA}$s, full-length topoisomerase I analogues were obtained by read-through of the UAG codon (Figure 5, lanes 3−16). Despite the lower level of expression for full-length human topoisomerase I analogues **8** and **9**, which had afforded almost no read-through during the

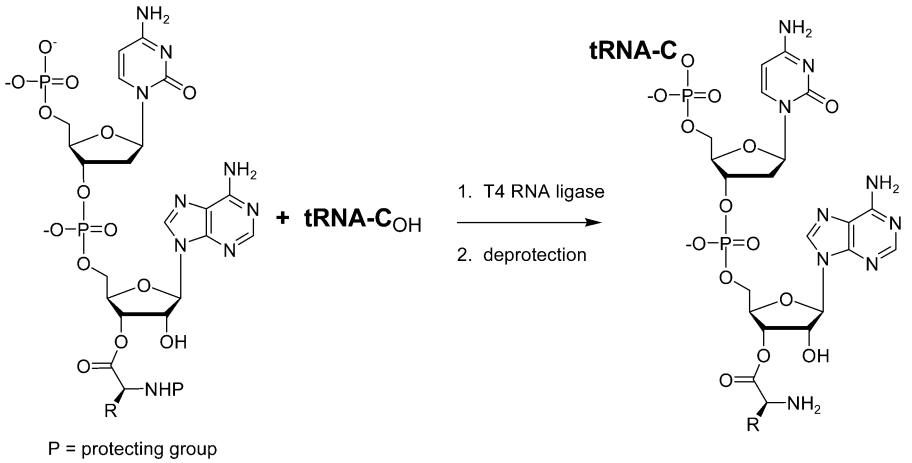

FIGURE 4: Chemical aminoacylation of misacylated suppressor tRNAs.

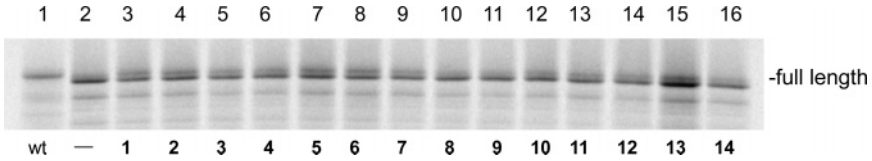

FIGURE 5: In vitro synthesis of human topoisomerase I analogues modified at position 723: lane 1, wild-type topoisomerase I; lane 2, truncated topoisomerase I-722 (no suppressor tRNA); and lanes 3−16, modified topoisomerase I analogues containing amino acids **1**−**14** at position 723, respectively. The syntheses were carried out in rabbit reticulocyte lysate at 30 °C over a period of 2 h. Purification of the modified topoisomerase I analogues was carried out by heparin agarose chromatography.

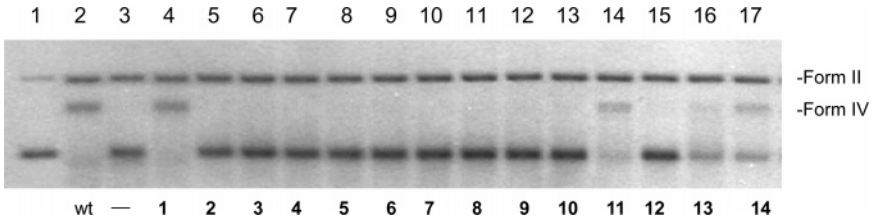

FIGURE 6: Human topoisomerase I-mediated DNA relaxation assay: lane 1, plasmid pSP64 alone; lane 2, DNA with wild-type topoisomerase I; lane 3, DNA with truncated topoisomerase I; and lanes 4−17, DNA with modified topoisomerase I analogues containing amino acids **1**−**14** at position 723, respectively. The assay was carried out in 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 250 ng of supercoiled pSP64 plasmid DNA, and 1.5 μL of (modified) topoisomerase I translation mixture. After incubation at 37 °C for 4 h, the incubation mixtures were analyzed by electrophoresis on a 1% agarose gel.

elaboration of modified vaccinia topoisomerase I analogues (*21*), all human topoisomerase I analogues were expressed at a level that permitted further study.

Assay for Topoisomerase I Function. To study the activities of the modified human topoisomerase I analogues, a DNA relaxation assay was used to compare their abilities to relax supercoiled DNA plasmid. The relaxation activity was determined by incubation of 1.5 μL of the crude translation mixture with 250 ng of supercoiled DNA plasmid in a 10 μL reaction mixture at 37 °C for 4 h. 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 DNA plasmid (form IV) from the supercoiled DNA plasmid (form I) and (adventitious) nicked DNA plasmid (form II). As shown in Figure 6, the DNA relaxation activity was essentially eliminated by replacement of the tyrosine OH group with H (**2**), NH_2 (**3**), SH (**4**), I (**5**), and CH_2NH_2 (**6**) groups (Figure 6, lanes 5−9). In addition, any modification that changed the position of the nucleophilic OH group on the active site tyrosine relative to the protein backbone (**7** and **8**) or which added a phenyl ring (**9** and **10**) also resulted in a loss of enzyme activity (Figure 6, lanes 10−13). Only those modifications containing tyrosine analogues (**11**, **13**, and **14**) having an −OH group in the "normal" position relative to the peptide backbone exhibited detectable relaxation activity (Figure 6, cf. lanes 14, 16, and 17), and even one topoisomerase I containing an analogue of this type (**12**) lacked detectable activity.

For those active human topoisomerase I analogues, each enzyme was purified by heparin agarose chromatography and the concentrations were quantified as described in Experimental Procedures. To compare the relative activities of wild-type human topoisomerase I with those of the three active topoisomerase I analogues (**11**, **13**, and **14**), the concentration dependence and time dependence of the relaxation assays were determined as shown in Figures 7 and 8. In addition, the rate constants (k_{obs}) for DNA relaxation were also determined for each of the human topoisomerase I analogues and are summarized in Table 1. DNA relaxation activity was found to be greatest for wild-type topoisomerase I (i.e., having tyrosine at position 723). For the topoisomerase analogues, activity decreased in the following order when

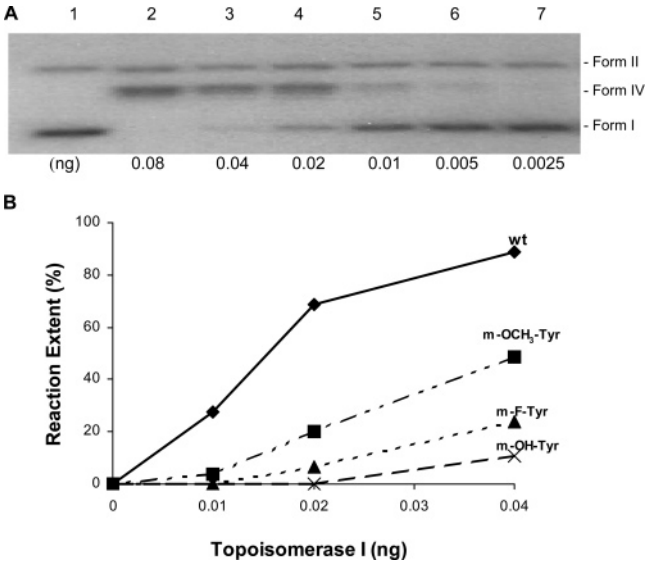

FIGURE 7: (A) Electrophoretic analysis of the concentration dependence of human topoisomerase I-mediated DNA relaxation. The assay was carried out by incubation with 0.08, 0.04, 0.02, 0.01, 0.005, or 0.0025 ng of wild-type topoisomerase I with 250 ng of supercoiled plasmid pSP64 DNA at 37 °C for 4 h: lane 1, plasmid pSP64 DNA alone; and lanes 2−7, plasmid pSP64 DNA in the presence of different amounts of wild-type topoisomerase I. (B) Concentration dependence of DNA relaxation by wild-type human topoisomerase I and three analogues modified at position 723.

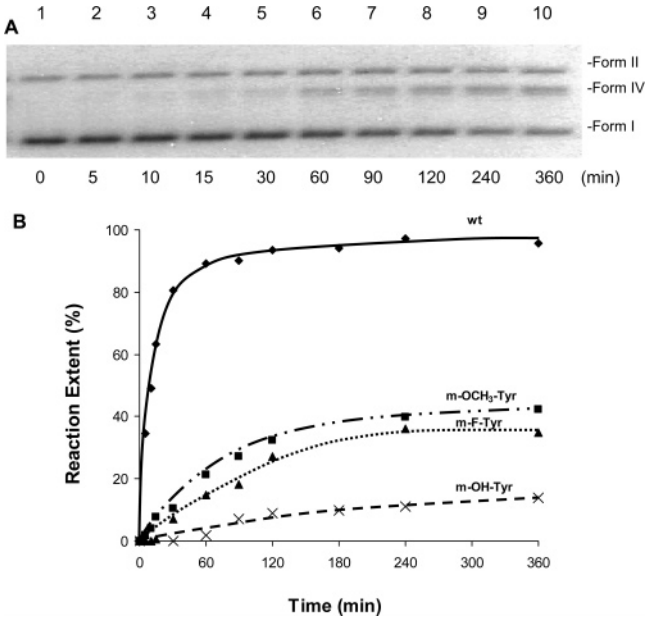

FIGURE 8: (A) Electrophoretic analysis of the time course of DNA relaxation mediated by a human topoisomerase I analogue containing **14** at position 723. The reaction was carried out at 37 °C as indicated in the legend of Figure 6, but using 0.04 ng of (modified) topoisomerase I. Aliquots were removed at the times indicated, and the reaction was quenched by adding 1% sodium dodecyl sulfate. (B) Time course of DNA relaxation comparing the activity of wild-type human topoisomerase I and three analogues modified at position 723.

these tyrosine derivatives were substituted at position 723 of the enzyme: **14** > **13** > **11**. As observed previously for vaccinia topoisomerase I (*21*), these findings seem consistent with the altered nucleophilicity of the *p*-tyrosine OH group in the active site. Thus, while each of the meta-substituted analogues was less active than the wild-type enzyme, the electron-donating OCH_3 substituent adjacent to the OH group resulted in the most active topoisomerase I analogue. It also seems possible that the substituent ortho to the tyrosine OH group may alter enzyme function via H-bonding, a factor that could contribute to the activity of those enzyme analogues containing the OCH_3 (**14**), F (**13**), and OH (**11**) substituents.

Table 1: Kinetic Parameters for DNA Relaxation Mediated by Analogues of Human Topoisomerase I Modified at Position 723[a]

amino acid at position 723	k_{obs} ($\times 10^5$ s^{-1})
Tyr (wild type) (**1**)	70 ± 3
m-OH-Tyr (**11**)	0.7 ± 1
m-F-Tyr (**13**)	3 ± 1
m-OCH_3-Tyr (**14**)	6 ± 1

[a] Rate constants were obtained under pseudo-first-order reaction conditions using a large excess of DNA substrate.

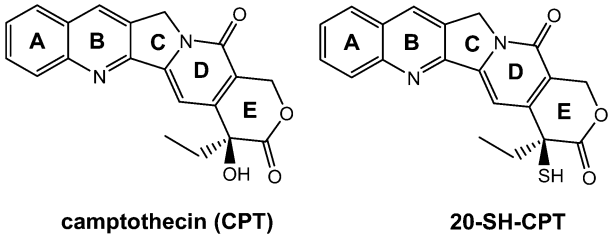

FIGURE 9: Structure of camptothecin and its analogue 20-thiocamptothecin.

Inhibition of Modified Human Topoisomerase I Analogues by Camptothecin. The ability of camptothecins (CPTs) to inhibit human topoisomerase I-mediated DNA relaxation has been investigated by using camptothecin and its analogue 20-thiocamptothecin (Figure 9) to determine their effects on both the wild-type enzyme and the most active analogue having **14** at active site position 723. After incubation at 37 °C for a predetermined time, the reactions were analyzed by 1% agarose gel electrophoresis as shown in Figure 10. In the absence of inhibitors, 250 ng of DNA plasmid could be completely relaxed with wild-type topoisomerase I within 1 h, but relaxation was incomplete for the modified enzyme even after 6 h because of its lower activity (Figure 10, lanes 1−6; cf. Table 1). In the presence of camptothecin, the course of DNA relaxation was inhibited for both the wild type and the modified enzymes (Figure 10, lanes 7−12). Although no obvious inhibition was observed for DNA relaxation by wild-type topoisomerase I, 20-thio CPT was shown to inhibit the relaxation of DNA topoisomerase I analogue **14** (Figure 10, lanes 13−18). After quantification by densitometry, the time courses of the reactions were also determined (Figure 11). By comparison of the inhibitory effect of CPTs on the wild-type enzyme and analogue **14**, it was shown that the modified topoisomerase I was more sensitive to the inhibitors, in parallel with its significantly decreased enzyme activity.

DISCUSSION

By utilizing "chemically" misacylated suppressor tRNAs to effect the read-through of a nonsense codon (*21*, *31*−*33*) at position 723, tyrosine and 13 different tyrosine analogues were incorporated into the active site of human topoi-

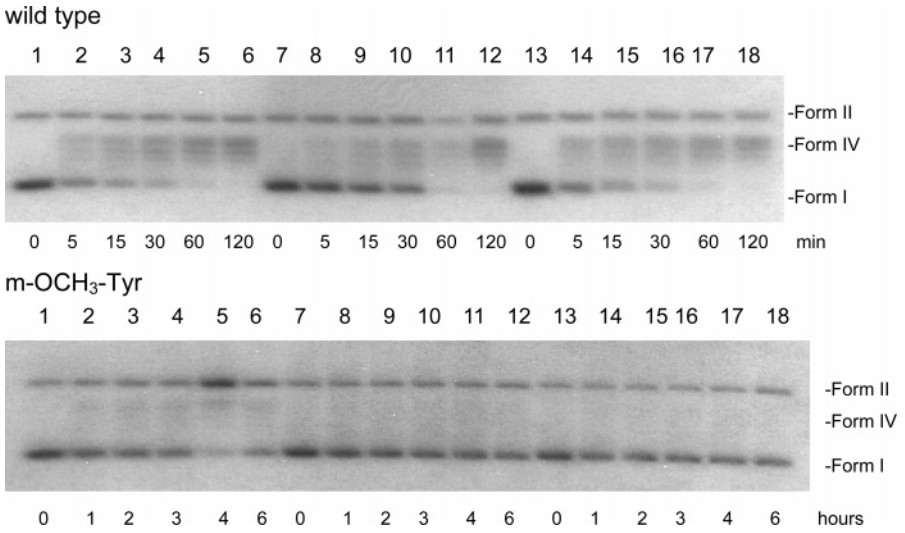

FIGURE 10: Electrophoretic analysis of the time course of inhibition by CPT and 20-thio CPT of human topoisomerase I-mediated DNA relaxation. The reaction was carried out by incubation of 0.04 ng of wild-type topoisomerase I or its analogue containing **14** with 250 ng of plasmid pSP64 DNA at 37 °C for the times indicated: lanes 1−6, in the absence of inhibitors; lanes 7−12, in the presence of 500 μM CPT; and lanes 13−18, in the presence of 500 μM 20-thio CPT.

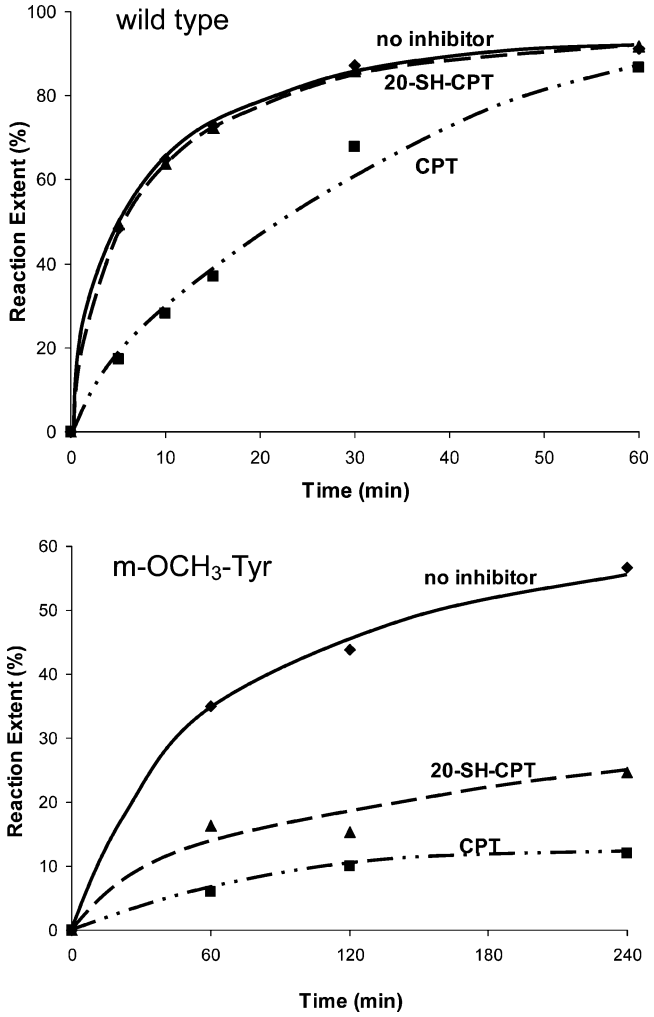

FIGURE 11: Time course of inhibition by CPT and 20-thio CPT of human topoisomerase I-mediated DNA relaxation for wild-type human topoisomerase I and its analogue containing **14** at position 723.

somerase I; this permitted the study of the structure−function relationships for this tyrosine moiety in the transesterification reaction. It was found that only the topoisomerase I analogues having tyrosines **11**, **13**, and **14** were active, i.e., those having the phenolic −OH group in the normal position relative to the protein backbone (Figure 6, lanes 14, 16, and 17). Modifications that replaced the nucleophilic tyrosine OH (**1**) group with NH_2 (**3**), SH (**4**), and I (**5**) groups, or that changed the spatial orientation of the nucleophilic OH group (**7** and **8**), essentially eliminated topoisomerase I function (Figure 6, lanes 6−13). For the active analogues (**11**, **13**, and **14**), the order of relaxation activity was as follows: **1** > **14** > **13** > **11** (which may be explained by the electronic effects and H-bonding characteristics of substituents in the meta position of the aromatic ring). The barely observable activity for analogue **12** is consistent with the impaired nucleophilicity of the phenolic OH group resulting from the strong electron-withdrawing effect of the adjacent NO_2 group. It may be noted that this analogue supported the activity of the analogous modified vaccinia topoisomerase I (*21*) but did so less well than those topoisomerase I analogues containing **11**, **13**, and **14**. The foregoing results observed with the modified human topoisomerase I analogues are thus consistent with those described previously for vaccinia topoisomerase I (*21*), which implies a strict requirement for the position of the nucleophilic −OH group in both enzymes.

Vaccinia and human topoisomerases I, two extensively studied type IB topoisomerases, have been shown to be distinct from each other in several ways even though they share the same basic mechanism of DNA binding, cleavage, and religation (*11*−*13*). Vaccinia topoisomerase I is the smallest type IB topoisomerase (31 kDa) (*34*) having a stringent requirement for a DNA substrate with the sequence 5′(C/T)CCTTp$^{\downarrow}$ in the transesterification reaction (*35*−*37*). While human topoisomerase I is a larger and more complicated enzyme (97 kDa) (*38*), it exhibits less specific sequence selectivity for its DNA substrate (*39*). The divergence of these two enzymes has been identified in their mechanism of binding and cleavage of DNA substrates (*15*−*20*). Further, camptothecin, a clinically used anticancer agent (*40*), recognizes human topoisomerase I and inhibits the enzyme-mediated DNA religation reaction (*8*−*10*) but has no effect on vaccinia topoisomerase I.

Since recent crystallographic studies have revealed that the catalytic tyrosines in human and vaccinia topoisomerases I differ to some extent despite the remarkable similarity of both enzymes (*41*), it seemed reasonable to anticipate differential effects of the replacement of the active site tyrosines in these enzyme with different unnatural tyrosine analogues having varying steric, electronic, and regiochemical features. However, the results presented here demonstrate surprisingly little difference in the effects on these two enzymes resulting from modification of their catalytic tyrosines, at least at the level of DNA relaxation ability. These results highlight the crucial role of tyrosine in both enzymes, which must be highly conserved to support the enzyme activity (*42*, *43*). In addition, the apparently increased sensitivity to CPTs after modification of human topoisomerase I at position 723 further emphasizes the critical role of the active site tyrosine (Figures 10 and 11). These conclusions have been reached using a topoisomerase I-mediated DNA relaxation reaction, which incorporates both topoisomerase I-mediated DNA cleavage and religation reactions. To further investigate the role of the active site tyrosine, it is clearly of interest to evaluate each individual enzyme by studying the DNA cleavage and religation activities separately after modification of the active site tyrosine with different unnatural amino acids. The possible effects of active site modification on the sequence selectivity of DNA cleavage also require study but would require access to amounts of modified topoisomerase I analogues significantly greater than that permitted by the level of protein expression realized in this study.

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