Identification of a Minimal Functional Linker in Human Topoisomerase I by Domain Swapping with Cre Recombinase[†]

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Received January 7, 2008; Revised Manuscript Received April 30, 2008

ABSTRACT: Cellular forms of type IB topoisomerases distinguish themselves from their viral counterparts and the tyrosine recombinases to which they are closely related by having rather extensive N-terminal and linker domains. The functions and necessity of these domains are not yet fully unraveled. In this study we replace 86 amino acids including the linker domain of the cellular type IB topoisomerase, human topoisomerase I, with four, six, or eight amino acids from the corresponding short loop region in Cre recombinase. *In vitro* characterization of the resulting chimeras, denoted Cropos, reveals that six amino acids from the Cre linker loop constitute the minimal length of a functional linker in human topoisomerase I.

Human topoisomerase I (topo I)[1] plays an important cellular role by relaxing superhelical strain arising in the genome during DNA tracking processes such as transcription and replication (*1*). The enzyme exerts this function by introducing transient single-strand breaks in the DNA backbone. It uses an active site tyrosine residue as a nucleophile to attack the scissile phosphodiester in the DNA, generating a covalent 3′-phosphotyrosyl cleavage intermediate and a 5′-OH DNA end. DNA relaxation is facilitated by the generated 5′-OH end rotating around the uncut strand in a manner that is controlled by the covalently bound enzyme before the nick is resealed in a reaction where the 5′-OH acts as the nucleophile and attacks the phosphotyrosyl linkage (*2, 3*). This chemical reaction scheme is shared by all members of the type IB topoisomerase family including viral, mitochondrial, and nuclear species of topo I (*3*), as well as by the tyrosine recombinases, such as Flp and Cre (*4, 5*), which uses the basal type IB mechanism of action to facilitate recombination between short well-defined DNA sequences.

Despite the fact that members of the type IB topoisomerase/tyrosine recombinase superfamily display a strikingly low homology in primary sequence (*3, 4, 6–8*), the analogous chemical reactions catalyzed by these enzymes are reflected in the extended structural homology between all crystallized members of the superfamily (*4, 9–11*). In particular, the spatial arrangements of the conserved RK(H/K)R(H/W) pentad motif and the catalytic tyrosine constituting the active site are superimposable within a few angstroms (*4*). Also, the overall structures of members of the superfamily share important features. Common to all crystallized topo Is and tyrosine recombinases is that the globular body of the enzymes form a bilobed clamp encompassing the DNA double helix. The clamp is formed by the core and C-terminal domain in type IB topoisomerases and by the N-terminal and C-terminal domains in tyrosine recombinases (*4, 9–14*). In all members of the superfamily the active site tyrosine is presented on an α-helix separated from the catalytic pentad by a linker region, which varies considerably in length from being a short loop region of only a few amino acids in enzymes such as Cre and vaccinia virus topo I (*4, 15*) to an extended domain such as the 76 amino acid (amino acids 636−712) coiled-coil structure in human topo I (*16*).

It is as yet unclear whether the coiled-coil linker domain of human topo I is strictly necessary for catalytic activity *per se*. The bulk part of structural and experimental data suggests that this domain, together with a rather extensive N-terminal domain (absent from viral topo I and the tyrosine recombinases), plays a specialized role in regulating the strand rotation step of catalysis and may be dispensable for cleavage/ligation activity. Hence, it has been reported that a human topo I variant lacking 40% of the linker domain retains catalytic activity although it relaxes negative supercoils in a rather uncontrolled manner (*17*).

Here, to further dissect the requirement put on the linker region of human topo I, the entire domain was replaced by the corresponding four, six, or eight amino acids of Cre

[†] This work was supported by the Carlsberg Foundation, the Danish Research Councils, the Danish Cancer Society, the Novo Nordisk Foundation, the Aase and Ejnar Danielsen Foundation, the Family Hede Nielsen Foundation, and the Krista and Viggo Petersen Foundation.

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[1] Abbreviations: topo I, topoisomerase I; HT, human topo I (amino acids 1−765); Cropo4, human topo I having residues 631−717 replaced by residues 314−317 of Cre; Cropo6, human topo I having residues 631−717 replaced by residues 312−317 of Cre; Cropo8, human topo I having residues 631−717 replaced by residues 312−319 of Cre; CPT, camptothecin; DTT, dithiothreitol; Me_2SO, dimethyl sulfoxide; EtOH, ethanol; PMSF, phenylmethanesulfonyl fluoride; EtBr, ethidium bromide.

10.1021/bi800031k CCC: $40.75
Published on Web 06/14/2008

recombinase. Cre, rather than vaccinia virus topo I, was chosen as the swapping partner for human topo I in these experiments, since Cre retains sensitivity toward the human topo I specific anticancer drug camptothecin (Supporting Information Figure S1), which is not the case for the wild-type vaccinia virus topo I (*18*). Therefore, with respect to the chemical steps of catalysis Cre may be regarded more alike human topo I than is vaccinia virus topo I. Chimeric human topo I, having the native linker domain, together with a few surrounding amino acids, replaced with linker regions of six or eight amino acids from the corresponding Cre sequence, showed DNA relaxation activity. In contrast, a chimera with a four amino acid linker region was unable to relax or cleave DNA although this mutant retained the ability to bind DNA. Taken together, the presented results suggest that a region of six amino acids is the minimum functional linker length in human topo I.

MATERIALS AND METHODS

Reagents and Enzymes. Me_2SO and CPT were from Sigma-Aldrich. CPT was dissolved in 99.9% Me_2SO at 20 mM and stored at −20 °C. Oligonucleotides and primers were from DNA technology and the pYX213 plasmid was from RD systems.

Construction of Chimeric Enzymes. Amino acids 631−717 of human topo I corresponding to the linker domain and a few surrounding residues (swap points determined based on structural homology to Cre) were replaced by amino acids 314−317, corresponding to the linker region of Cre, by a three-step polymerase chain reaction procedure using the internal primer sets (sense) 5′-GTG GAG CAC ATC AAT CTA CA-3′ and (antisense) 5′-GGT ATT GGT CCA GCC ACA AAG AAT TGC AAC AGC TC-3′ and (sense) 5′-GTG GCT GGA CCA ATA CCT CCA AAC TCA ATT ATC T-3′ and (antisense) 5′-CTC ATA GTC TTC ATC AGC C-3′ and the external primers (sense) 5′-CGG TCA GAA TTC GCC GAC ATG AGT GGG GAC CAC-3′ and (antisense) 5′-GTT GCA GAG CTC ACT CTA AAA CTC ATA GTC TTC ATC-3. The resulting polymerase chain reaction product was cloned into the *Eco*RI/*Sac*I fragment of the *Saccharomyces cerevisiae* expression vector pYX213, generating pYX213 Cropo4. An N-terminal GST tag was inserted in pYX213Cropo4. The GST tag was amplified from the pGEX-2TK vector using primers (sense) 5′-CTG CAG GGC AAT TGT TCA TGT CCC CTA TAC TAG G-3′ and (antisense) 5′-GGT TGG CAT GAA TTC CGG GGA TCC AAC AGA TGC-3′. The resulting PCR product was cloned into the *Eco*RI fragment of pYX213Cropo4, generating pYX213-GST-Cropo4 (pTC100).

The linker region of Cropo4 was extended by Cre residues 312−313 using primers (sense) 5′-GCA ATT CTT TGT GCT GGT GGC TGG ACC AAT ACC-3′ and (antisense) 5′-GGT CCA GCC ACC AGC ACA AAG AAT TGC AAC AGC-3′, generating pTC101, and by Cre residues 318−319 using primers (sense) 5′-CTG GAC CAA TGT AAA TAC CTC CAA ACT CAA TTA TC-3′ and (antisense) 5′-GTT TGG AGG TAT TTA CAT TGG TCC AGC CAC CAG-3′, generating pTC102. The linker extensions were all introduced using the quick change procedure (*19*). The Expand High Fidelity PCR system (Roche) was used in all PCR reactions. All constructed plasmids were sequenced using the DY-Enamic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech) or the BigDye Termination Cycle Sequencing kit (Applied Biosystems) and analyzed on the ABI3130 capillary Sequencing setup.

Protein Expression and Purification. The plasmids pTC100, pTC101, and pTC102 for expression of Cropo4, Cropo6, and Cropo8, respectively, were transformed into the yeast *S. cerevisiae* top1Δ strain RS190 (a kind gift from R. Sternglanz, State University of New York, Stony Brook, NY) by the lithium acetate procedure (*20*). Crude cell extract from 8 L of yeast culture expressing the enzymes was prepared. The proteins were purified essentially as described (*21, 22*). Crude cell extract was precipitated by addition of 80% $(NH_4)_2SO_4$ followed by 12 h of incubation at 4 °C. Precipitated protein was collected in the pellet by centrifugation at 10000 rpm for 20 min. This pellet was carefully dissolved in a standard buffer containing 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, 1 mM DTT, and 1 mM PMSF, diluting the pellet 63 times to ensure a final salt concentration of 100 mM. After filtration, the extract was loaded onto a 10 mL heparin−Sepharose column, which was pre-equilibrated by 10 volumes of buffer containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 10% glycerol, and 1 mM PMSF. The column was washed with 5 volumes of the same buffer. Finally, the protein was eluted using the standard buffer containing 1 M NaCl. Protein containing fractions were identified by SDS−polyacrylamide gel electrophoresis, pooled, and diluted to 140 mM NaCl using a buffer composite of 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM PMSF, and 1 mM DTT. The diluted protein was loaded on a prepacked 1.5 mL glutathione column (Amersham Pharmacia) by gravity flow, washed by 10 volumes of PBS, and eluted by addition of elution buffer (100 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 25 mM glutathione, 10% glycerol, 1 mM DTT). Purified proteins were stored at −20 °C after addition of glycerol to a final concentration of 50%. Each of the enzymes HT, Cropo4, Cropo6, and Cropo8 (500 ng) was run in a standard SDS−polyacrylamide gel to confirm purity (Figure 1C).

HT was expressed from pHT143 and purified as previously described (*23*).

Assay for DNA Relaxation Activity. Relaxation reactions were carried out in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM $CaCl_2$, and 5 mM $MgCl_2$ plus varying concentrations of NaCl, or with or without CPT as stated in the text. A total of 200 fmol of negatively supercoiled pBR322 was incubated with a 10-fold molar excess of enzyme at 37 °C for indicated time intervals and stopped by the addition of 0.2% (w/v) SDS. Subsequent to proteolytic digestion with 0.5 μg/mL proteinase K at 37 °C for 30 min, the products were separated in a 1% agarose gel. DNA was visualized by staining the gel with 0.35 μg/mL ethidium bromide, and the gel image was analyzed using the Bio-Rad Gel Doc-2000 system.

Quantification of DNA Relaxation Activity. For all graphical representations of relaxations, the relative DNA amount was quantified using the QuantityOne software and the relaxation activity calculated as 1 − (the amount of supercoiled substrate left after incubation)/(total DNA amount), and this value was plotted as a function of concentration or incubation time. The relaxation quantifications shown in

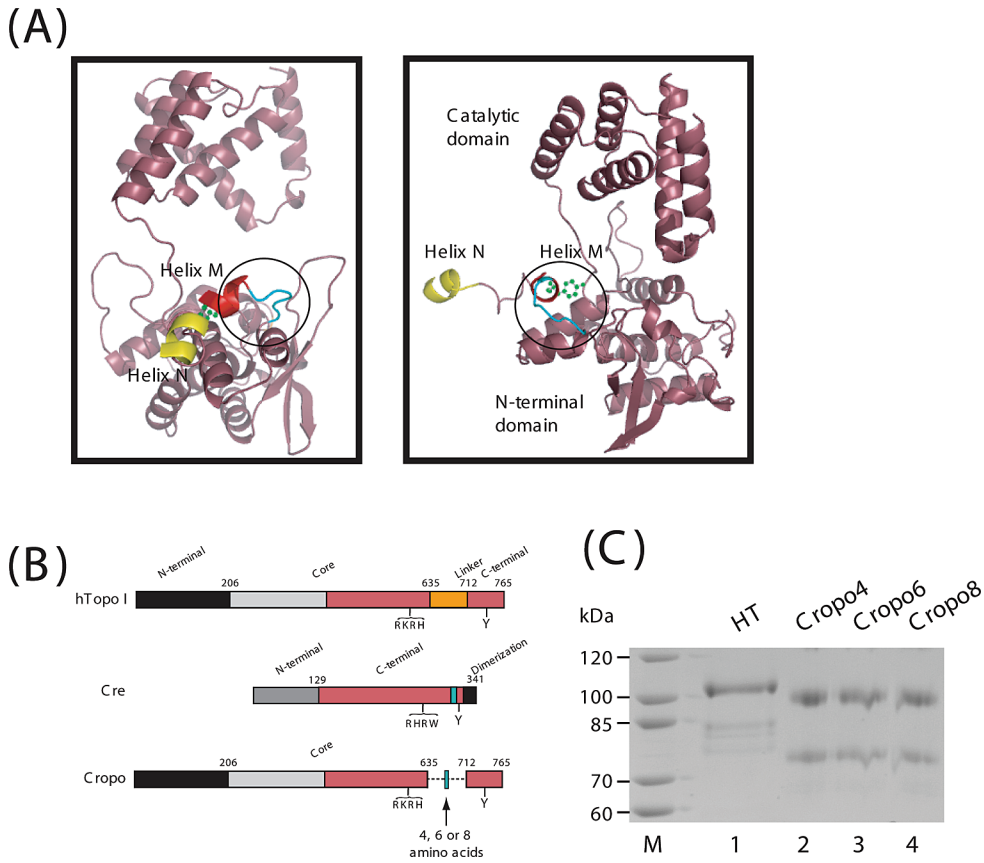

FIGURE 1: Chimeric enzymes characterized in this study. (A) Structure of Cre, viewed along (left panel) and perpendicular to (right panel) the DNA axis. The loop region investigated in the study is represented in cyan and indicated by a circle. Helix M is colored red, and helix N is shown in yellow. The figure was prepared using the PyMol molecular graphics system (from DeLano Scientific LLC) (*39*). (B) Schematic representation of the domain structure of human topo I, Cre, and the three chimeras Cropo4, Cropo6, and Cropo8. The conserved catalytic domains are shown in red. The human topo I linker domain is rendered in orange, and the corresponding region of Cre is shown in green. Domains which are unique to each enzyme are colored gray or black. (C) SDS−polyacrylamide gel electrophoresis analysis of 2 μg of purified HT (lane 1), Cropo4 (lane 2), Cropo6 (lane 3), and Cropo8 (lane 4) stained with Coomassie Brilliant Blue. The fast migrating bands in lanes 2−4 represent degradation products of the Cropos lacking the N-terminus of the enzymes. The marker lane is denoted M, and the protein sizes are shown to the left of the gel.

Figures 2, 3 and 4 were based on the accompanying representative relaxation gels.

Synthetic DNA Substrates. Oligonucleotides for construction of DNA binding substrates, suicide cleavage substrates, and DNA ligators were synthesized by DNA Technology on a model 394 DNA synthesizer from Applied Biosystems. The sequences of the substrates are as follows: OL100T, 5′-GCT ATA CGA ATT CGC TAT AAT TCA TAT GAT AGC GGA TCC AAA AAA GAC TTA GAA AAA AAA AAA GCT TAA GCA ACA TAT GGT ATC GTC GGA ATT CAA TGA G-3′; OL100B, 5′-CTC ATT GAA TTC CGA CGA TAC CAT ATG TTG CTT AAG CTT TTT TTT CTA AGT CTT TTT TGG ATC CGC TAT CAT ATG AAT TAT AGC GAA TTC GTA TAG C-3′; OL19, 5′-GCC TGC AGG TCG ACT CTA GAG GAT CTA AAA GAC TTA GA-3′; OL27, 5′-AAA AAT TTT TCT AAG TCT TTT AGA TCC TCT AGA GTC GAC CTG CAG GC-3′; OL50, 5′-AGA AAA ATT TTT GGC TTA-3′; OL51, 5′-TAA GCC AAA AAT TTT TCT AAG TCT TTT AGA TCC TCT AGA GTC GAC CTG CAG GC-3′; OL52, 5′- GCC TGC AGG TCG ACT CTA GAG GAT CTA AAA GAC TTA G-3′.

Purified oligonucleotides representing scissile strands (OL100T, OL19, and OL52) were 5′-radiolabeled by T4 polynucleotide kinase using [γ-^{32}P]ATP as the phosphoryl donor. To prevent ligation of the 5′-OH from the bottom strands (OL27 and OL51), these ends were 5′-phosphorylated with unlabeled ATP. Nonreacted ATP was removed by spin dialysis on a G-50 column. For hybridization, 10 pmol of each of the oligonucleotides constituting the substrate was mixed and slowly cooled to room temperature after heating to 85 °C, as previously described (*24*).

DNA Binding Assay. DNA binding of HT, Cropo4, Cropo6, Cropo8, and BSA was assayed by nitrocellulose filter binding as described previously (*22*). 5′-End-labeled DNA substrate (OL100T/OL100B) (125 fmol) was incubated with increasing amounts of protein as indicated in a binding buffer containing 50 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 5% glycerol, 1 mM EDTA, and 20 mM NaCl in a 100 μL reaction volume for 5 min at room temperature. Subsequently, the samples were applied to a 0.2 μm nitrocellulose filter under vacuum. To reduce nonspecific binding of the DNA substrate, the nitrocellulose filter was prewetted in binding buffer. After application of the samples, the filter was washed in 500 μL of binding buffer to remove unbound DNA substrate and dried. The relative amount of DNA retained on the filter was determined using a SF Molecular Dynamics PhosphorImager with the QuantityOne software, and the values were normalized to the amount of DNA retained in the presence of 1.2 μg of HT. The mean and

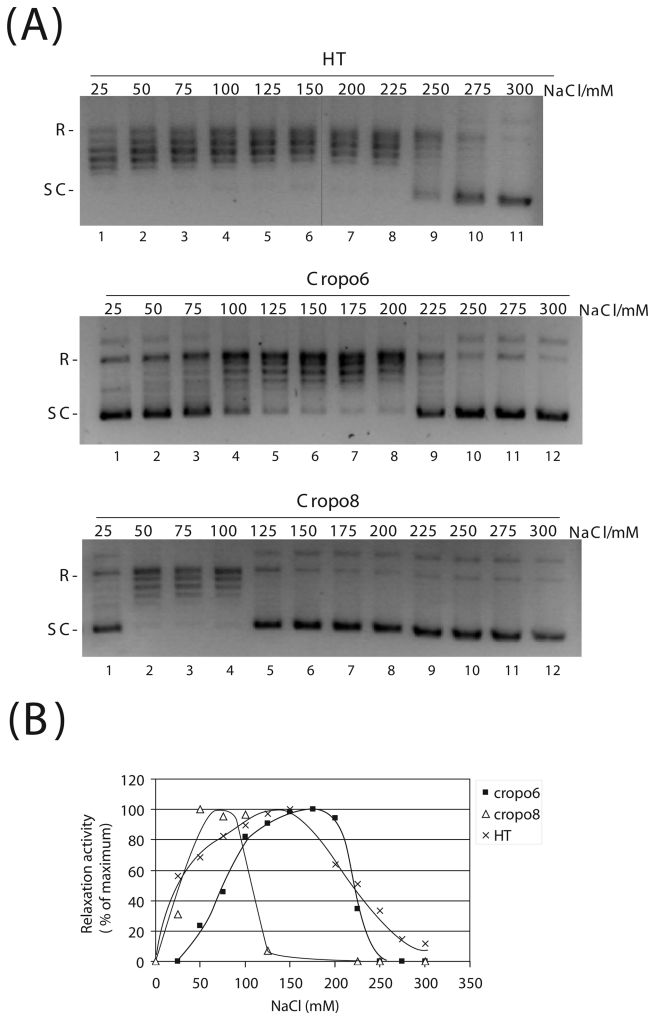

FIGURE 2: Optimal salt concentration for DNA relaxation activity by Cropo6 and Cropo8. (A) DNA relaxation activity of Cropo6 and Cropo8 at NaCl concentrations ranging from 25 to 300 mM as indicated on the figure. Key: SC, supercoiled plasmid; R, relaxed plasmid. (B) Graphical depiction of the DNA relaxation activity of Cropo6, Cropo8, and HT plotted as a function of the NaCl concentration. The data are normalized relative to the highest amount of activity reached by each enzyme.

standard deviation obtained from three independent experiments were plotted as a function of increasing protein amount.

Coupling to Hydrogen Peroxide. Nicking of plasmid by coupling of H_2O_2 was assayed by incubating recombinant enzyme with 300 ng of negatively supercoiled pBR322 in the absence or presence of 0.5% H_2O_2 (v/v) for 120 min at 37 °C. Reactions were performed in a 20 μL volume containing 5% Me_2SO, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.5 mM EDTA. Subsequent to termination by addition of 0.2% SDS, reaction products were analyzed on 1% agarose gels containing 0.35 μg/mL ethidium bromide. Coupling of the hydroxide nucleophile by each of the four enzymes was calculated as the appearance of the nicked form of plasmid relative to the total amount of plasmid and subtracted the background of nicked plasmid formed when no enzyme was added the reaction mixture. The quantifications were done using the QuantityOne software. For the graphical representation of the nicking reactions, the mean and standard deviation obtained from three independent experiments were plotted in a bar diagram.

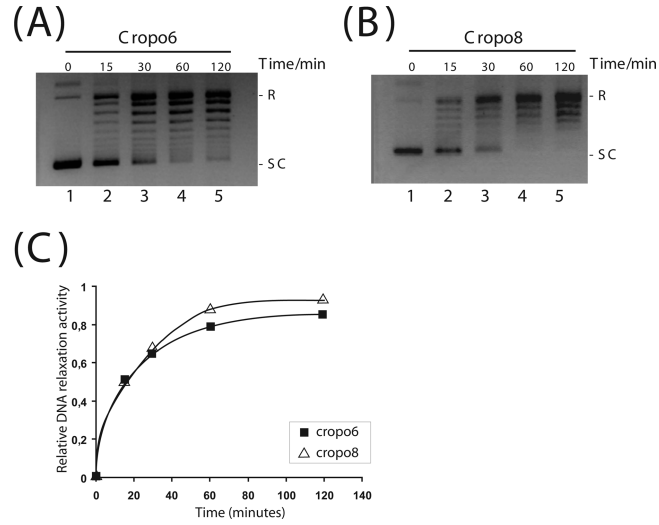

FIGURE 3: Characterization of the DNA relaxation activity of Cropo6 and Cropo8. Relaxation activities of Cropo6 and Cropo8 were assayed at their individual salt optima by incubation of 200 fmol of negatively supercoiled plasmid with an excess of purified enzyme at 37 °C for the indicated time intervals. (A) Representative gel showing the relaxation activity of Cropo6 assayed at 175 mM NaCl. (B) Representative gel showing the relaxation activity of Cropo8 investigated at 75 mM NaCl. Key: SC, supercoiled plasmid; R, relaxed plasmid. (C) Graphical representation of the relaxation activity quantified and calculated from the shown gels (A and B) as described in Materials and Methods and plotted as a function of incubation time.

Detection of Cleavage and Ligation Activity. Standard cleavage and ligation reactions were carried out by incubating 50 fmol of suicide DNA substrate OL19/OL27 or ligation substrate OL52 + OL50/OL51 with 500 fmol of enzyme in a 20 μL reaction volume at 37 °C for 120 min at standard conditions (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM $CaCl_2$). All reactions were stopped by addition of SDS to a final concentration of 0.2% (w/v).

The cleavage samples incubated with HT and all ligation samples were precipitated with 0.4 mM NaCl and 3 volumes of ethanol and subsequently digested with 1 mg/mL trypsin in 10 μL of 10 mM Tris-HCl (pH 7.5) and 5 mM EDTA for 30 min at 37 °C. The cleavage samples incubated with Cropo6 and Cropo8, however, were phenol/chloroform extracted to isolate the covalent protein−DNA complexes at the interphase between the phenol/chloroform phase and the water phase followed by dilution of the isolated interphases in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, before the samples were EtOH precipitated and trypsin digested.

Polyacrylamide Gel Electrophoresis. Succeeding trypsin digestion, cleavage and ligation samples were mixed with an equal volume of 80% (v/v) deionized formamide, 50 mM Tris−borate (pH 8.3), 1 mM EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene xyanol and applied to a 12% denaturing polyacrylamide gel. Radiolabeled DNA was visualized by a model SF Molecular Dynamics PhosphorImager.

RESULTS

Construction and Expression of the Chimeric Enzymes, Cropo4, Cropo6, and Cropo8. Residues 631−717 of human topo I, encompassing the coiled-coil linker domain (amino acids 636−712) and a few surrounding residues in the

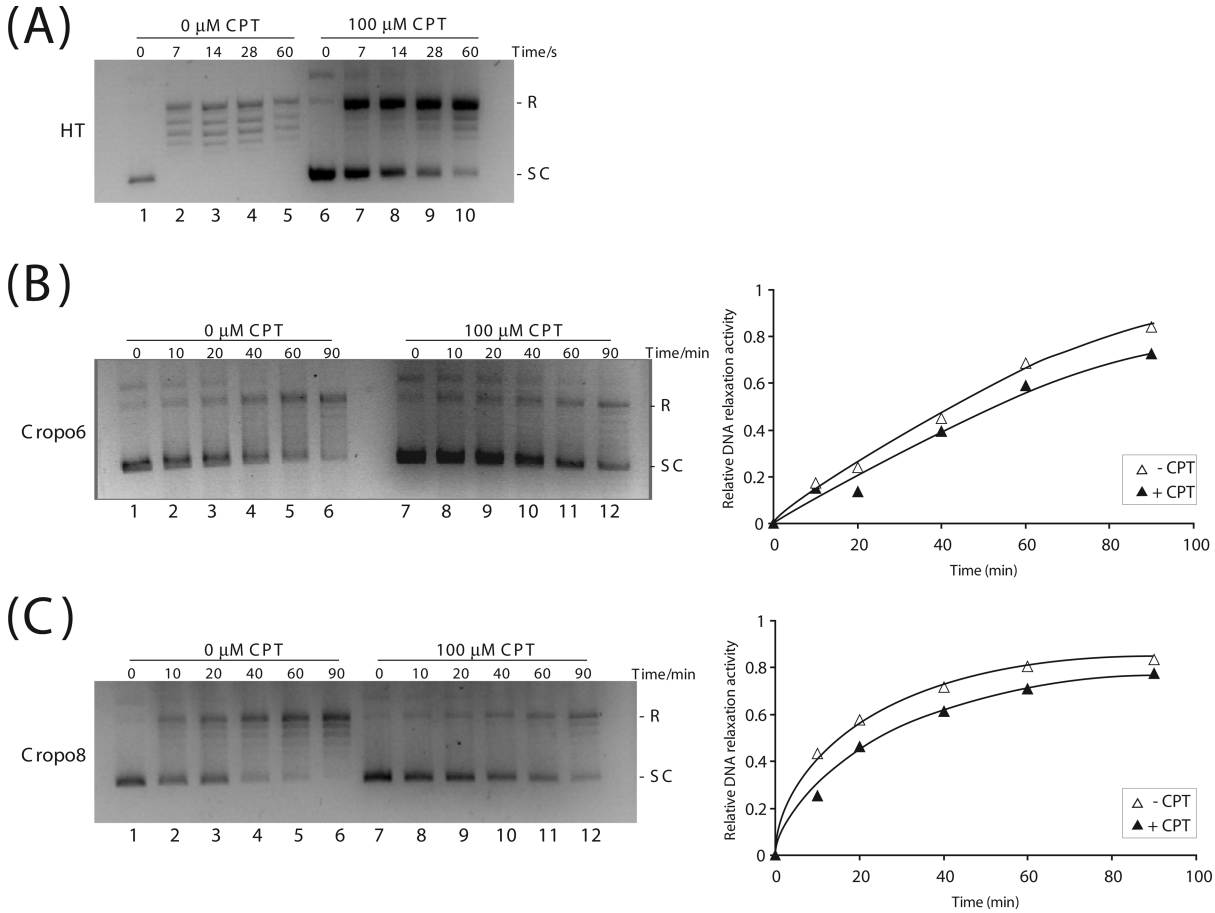

FIGURE 4: Effect of CPT on Cropo6- and Cropo8-mediated DNA relaxation. DNA relaxation reactions were carried out essentially as described for Figure 3. To assess the sensitivity toward CPT, 1 mM CPT dissolved in Me_2SO was added to the samples, resulting in a final concentration of 10% (v/v) Me_2SO. Control samples without CPT were added an equal volume of Me_2SO. (A) Representative gel showing the DNA relaxation activity of HT in the absence (lanes 1−5) or presence (lanes 6−10) of 100 μM CPT. Reactions were stopped after 0, 7, 14, 28, or 60 s. Representative gels showing the DNA relaxation activity of (B) Cropo6 or (C) Cropo8 in the absence (lanes 1−6) or presence (lanes 7−12) of 100 μM CPT. Samples were quenched after 0, 10, 20, 40, 60, or 90 min. The relative relaxation activities were quantified and calculated from the shown gels (B and C) as described in Materials and Methods, plotted as a function of incubation time and shown in the right panels of the figure. The relative DNA relaxation activity of HT is not plotted since the DNA was fully relaxed within the first 7 s of the reaction. Key: SC, supercoiled plasmid; R, relaxed plasmid.

enzyme, were replaced by varying lengths of the corresponding loop region of Cre including residues 314−317, 312−317, or 312−319 to generate the three chimeric enzymes denoted Cropo4, Cropo6, or Cropo8 in the following. The design of the chimeras including the determination of swapping points was based upon the crystal structures of human topo I and Cre (Figure 1A,B) in an attempt to cause minimal folding disturbances in the chimeric enzyme. An N-terminal GST tag was inserted to allow protein purification independent upon protein characteristics. Cropo4, Cropo6, and Cropo8 as well as wild-type human topo I (HT) were expressed in yeast *S. cerevisiae* and purified to homogeneity as described in the Materials and Methods section (Figure 1C).

Identification of the Minimal Linker Length Required for Human Topo I Relaxation Activity. Relaxation activities of the purified Cropo4, Cropo6, and Cropo8 were tested and compared to the HT relaxation activity in the presence of NaCl concentrations ranging from 0−300 mM (Figure 2A). Negatively supercoiled plasmid was incubated with a 10-fold molar excess of each of the four enzymes at 37 °C, and the reaction products were analyzed by agarose gel electrophoresis. For the Cropos, incubation was continued for 120 min while relaxation mediated by HT was stopped after 7 s. The different incubation times were necessary due to very different DNA relaxations rates of the Cropo variants and HT (see Figure 3).

No activity of Cropo4 was observed at any of the investigated NaCl concentrations (Supporting Information Figure S2), whereas Cropo6, Cropo8, and HT all exhibited NaCl-dependent DNA relaxation activity as evident from the graphic depiction of the results in Figure 2B. In agreement with previous reports, the NaCl optimum for HT was 150 mM, whereas the two Cropo variants exhibit remarkably different NaCl optima: 175 mM for Cropo6 and 75 mM for Cropo8. Therefore, 150, 175, or 75 mM NaCl was added to the reaction mixtures when assaying DNA relaxation activity of HT, Cropo6, or Cropo8, respectively, in the following.

Since Cropo6 and not Cropo4 exhibited DNA relaxation activity, we concluded the six amino acid loop region from Cre to be the minimal linker necessary for DNA relaxation activity. Cropo4 was excluded from further assaying of DNA relaxation activity.

Characterization of the Relaxation Activity of Cropo6 and Cropo8. The relaxation activities of Cropo6 and Cropo8 were further characterized in the presence of $MgCl_2$, which is dispensable but stimulatory for the relaxation activity of human topo I. Except for the addition of $MgCl_2$ to the

reaction buffer, the experiments were carried out essentially as described above. Reactions were stopped after increasing time intervals as stated in Figure 3 and analyzed by agarose gel electrophoresis. Both enzymes relaxed the supercoiled DNA plasmid at a relatively slow rate compared to HT, which completed the reactions within 7 s under comparable incubation conditions (see Figure 4A). For both Cropo6 and Cropo 8, the maximal degree of relaxation was reached after 60 min of incubation where approximately 80% and 90%, respectively, of the supercoiled plasmids were converted into relaxed topoisomers (calculated as described in Materials and Methods) (Figure 3A, lane 4; Figure 3B, lane 4; and Figure 3C, graphical representation).

The human topo I targeting anticancer drug camptothecin (CPT), best known for inhibiting the religation step of catalysis, also blocks the relaxation step by a mechanism believed to involve stalling of human topo I in a conformation which sterically prevents strand rotation (*25*). Sensitivity of HT, Cropo6, and Cropo8 toward CPT in relaxation was investigated by incubating each of the three enzymes with supercoiled plasmid in the presence or absence of 100 μM CPT at 37 °C for indicated time intervals, prior to gel electrophoretic analysis of the reaction products (Figure 4). Consistent with previous reports, relaxation by HT was inhibited more than 10-fold upon the addition of CPT (Figure 4A, compare lanes 2 and 10). In contrast, both Cropo6 and Cropo8 were inhibited approximately 2-fold by the drug in DNA relaxation (Figure 4B, left panel, compare lanes 12 and 5, and Figure 4C, left panel, compare lanes 12 and 4. Note that the amount of DNA in samples containing CPT appears higher than the DNA amount in samples without CPT as estimated from EtBr staining of the gel. This is merely an artifact caused by CPT affecting the EtBr staining of DNA. The right panels of Figure 4B,C show graphical representations of the results). The insensitivity of the two Cropo variants toward CPT in DNA relaxation activity was unlikely a cause of inefficient drug binding as such, since DNA ligation activity by both Cropos was readily inhibited by CPT (data not shown).

Coupling of Hydrogen Peroxide by HT, Cropo4, Cropo6, and Cropo8. The lack of detectable relaxation activity of Cropo4 might be a consequence of a malfunctional (too short) linker region leading to a simple displacement of the active site tyrosine in the catalytic pocket, or it may reflect general misfolding of the enzyme. Similar considerations may be put forward to explain the decreased relaxation activity of Cropo6 and Cropo8 relative to HT. These possibilities are difficult to distinguish experimentally. However, the ability of type IB topoisomerases and tyrosine recombinases to utilize hydrogen peroxide as a substitute for the natural nucleophile in the cleavage and/or ligation reactions (see Figure 5A for schematic illustration of the relevant reactions) can be exploited to address the issue further (*24, 26–28*). In the *trans* cleaving Flp, cleavage (or ligation) by hydrogen peroxide, for instance, proceeds in the absence of the active site tyrosine but does require activation of the scissile phosphate (or of a substitute for the phosphotyrosine bond) by the catalytic pentad (*29, 30*). Ligation of hydrogen peroxide by the *cis* cleaving type IB topoisomerases succeeds cleavage and seems to require a complete functional catalytic site (until now active site tyrosine-independent utilization of hydrogen peroxide by wild-type human topo I has not been observed; unpublished results) (*21, 26, 27*). Hence, based on the available data it seems reasonable to assume that the minimal requirements for utilization of hydrogen peroxide in either the cleavage or

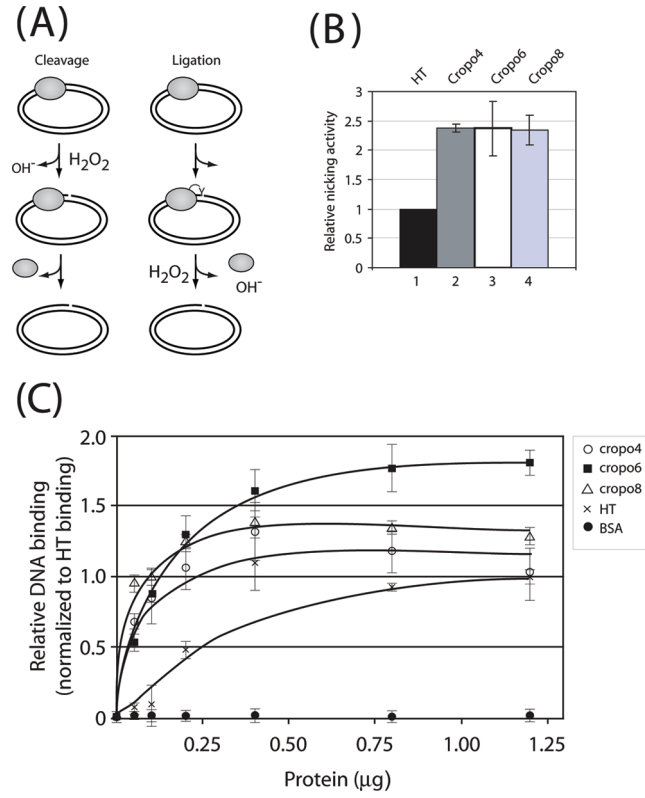

FIGURE 5: Hydrogen peroxide nicking and DNA binding by Cropo4, Cropo6, and Cropo8. (A) The abilities of HT, Cropo4, Cropo6, and Cropo8 to nick negatively supercoiled DNA by coupling to hydrogen peroxide were addressed. Schematical depiction of hydrogen peroxide mediated cleavage and ligation reactions are shown. In the cleavage reaction, hydrogen peroxide substitutes for the tyrosine in the active site of the enzyme and creates a nick in the plasmid by a nucleophilic attack. Since no phosphotyrosine bond between the enzyme and the plasmid is created during this reaction, the enzyme is not covalently attached to the DNA, and the result of the reaction is a nicked plasmid. In the hydrogen peroxide mediated ligation reaction, the plasmid is cleaved by the enzyme in a normal cleavage reaction, and the enzyme is thus covalently attached to the DNA prior to reaction with hydrogen peroxide. Instead of the free DNA hydroxyl end performing a nucleophilic attack on the phosphotyrosine bond to restore the DNA strand, hydrogen peroxide performs the nucleophilic attack, and the end result of the reaction is release of the enzyme from the DNA, which remains nicked. (B) 200 fmol of plasmid was reacted with HT, Cropo4, Cropo6, or Cropo8 in the presence of 0.5% (v/v) hydrogen peroxide for 120 min at 37 °C. Reaction products were analyzed by agarose gel electrophoresis. Nicking activities were measured as the relative amounts of plasmid converted to the nicked form. The background chemical nicking was subtracted, and the values were normalized relative to the nicking activity observed for HT. (C) DNA binding of Cropo4, Cropo6, and Cropo8. Binding of HT, Cropo4, Cropo6, Cropo8, and BSA was assayed by incubating increasing amounts of protein with a 5′-end-labeled substrate DNA (OL100T/OL100T) for 5 min at room temperature. Enzyme-bound DNA was separated from unbound DNA by filtration through a nitrocellulose membrane, which specifically retains protein−DNA complexes. The BSA sample was included to demonstrate that unspecific DNA binding was negligible. The amount of radiolabeled DNA retained on the filter was quantified on a SF Molecular Dynamics PhosphorImager, normalized to the amount of DNA retained in the presence of 1.2 μg of HT and plotted as a function of the protein concentration.

the ligation step of catalysis are DNA binding and assembly of a functional catalytic pentad.

Therefore, to test whether the three Cropo variants retain a functional catalytic pentad and bind to DNA in an active conformation, we compared their ability to utilize hydrogen peroxide as a nucleophile to introduce DNA nicking with that of HT. In the experimental setup, supercoiled plasmid was incubated with a 10-fold molar surplus of HT, Cropo4, Cropo6, or Cropo8 in the presence of hydrogen peroxide as described in Materials and Methods, and the amount of supercoiled plasmid converted to a nicked form was determined by gel electrophoretic analysis. The graphical representation of the results (Figure 5B) demonstrates that all three chimeras catalyzed coupling of hydroxide with equal efficiency (columns 2, 3, and 4), which was more than 2-fold higher than the hydroxide coupling activity of HT (Figure 5B, column 1). This result suggests that all three Cropo variants bind DNA in an active conformation. To further confirm the DNA binding capacity of Cropo4, Cropo6, and Cropo8, the ability of the enzymes to retain a radiolabeled double-stranded 100-mer substrate on a nitrocellulose membrane was compared to that of HT using a standard filter binding assay. As evident from Figure 5C, comparable amounts of radiolabeled substrate were retained on the filter upon incubation with increasing amounts of either Cropo4, Cropo6, Cropo8, or HT. As a negative control we used the non-DNA binding protein, BSA, which did not retain any DNA on the filter.

Cleavage and Ligation Activity of Cropo Variants. The ability of the relaxation-competent enzymes Cropo6 and Cropo8 to cleave and ligate DNA was addressed using synthetic DNA substrates which allow the cleavage and ligation steps of catalysis to be investigated separately (*31*). The substrate (OL19/OL27) used for assaying cleavage allows covalent attachment of the enzyme to the 3′-end of the scissile strand (OL19). The religation reaction, which normally succeeds cleavage, however, is prevented due to diffusion of the 5′-OH end of the trinucleotide (5′HO-AGA) generated during cleavage (Figure 6A, right panel). Cleavage products were visualized by 5′-radiolabeling of the scissile strand (OL19), and ligation to the noncleaved strand (OL27) was prevented by 5′-cold phosphorylation. Ligation was assayed using a reminiscent substrate (OL52 + OL50/OL51) where ligation to OL50 succeeded cleavage of OL52 followed by diffusion of the dinucleotide (5′-HO-AG) generated during cleavage (Figure 6B, right panel). The ligation products were visualized by 5′-radiolabeling of the scissile strand (OL52).

To assay DNA cleavage, a 50-fold molar surplus of HT, Cropo6, or Cropo8 was incubated with radiolabeled OL19/OL27 for 120 min at 37 °C. The reactions were terminated by the addition of SDS, and samples incubated with Cropo6 or Cropo8 were phenol extracted to isolate the cleavage complexes from the interphase (to enhance the sensitivity of the assay) prior to EtOH precipitation and trypsin digestion. The sample incubated with HT was EtOH precipitated and trypsin digested without prior phenol extraction. All samples were analyzed by denaturing polyacrylamide gel electrophoresis. As apparent from Figure 6A, left panel, HT, Cropo6, and Cropo8 all mediated cleavage of the synthetic DNA substrate (lanes 2, 3, and 4, respectively), whereas no activity was detected for Cropo4 (data not shown). Note,

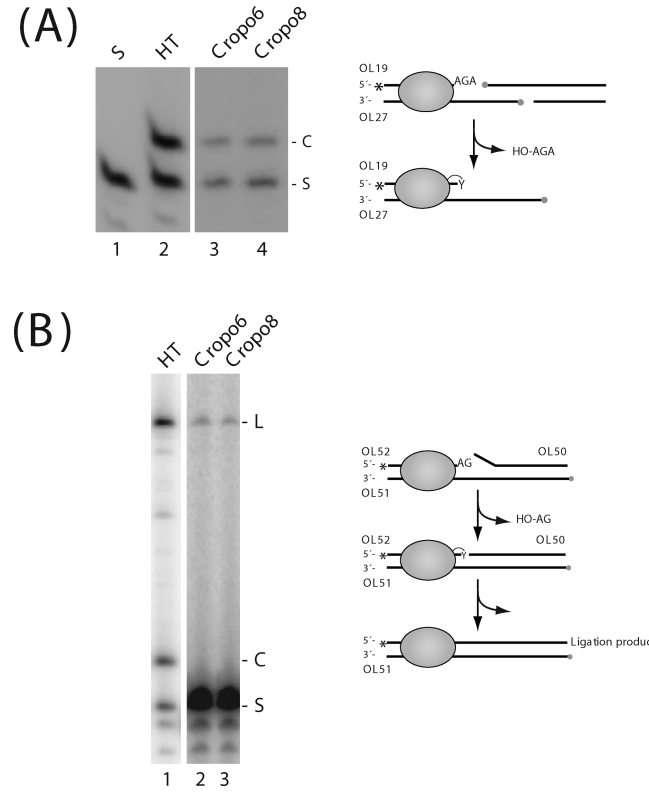

FIGURE 6: Cleavage and ligation activity of Cropo6 and Cropo8. (A) Cleavage activity of Cropo6 and Cropo8. The cleavage reaction is schematically depicted in the right panel. The substrate (OL19/OL27) used for assaying cleavage allows covalent attachment of the enzyme to the 3′-end of the 5′-radiolabeled scissile strand (OL19) by cleaving off a trinucleotide. Religation is prevented due to dissociation of the trinucleotide. Reactions were carried out by incubating the radiolabeled DNA substrate (lane 1) with HT (lane 2), Cropo6 (lane 3), or Cropo8 (lane 4) at 37 °C for 120 min before quenching the reaction by SDS. Cleavage products were analyzed by denaturing gel electrophoresis and subsequent autoradiography. Due to a trypsin-resistant peptide of a molecular mass corresponding to five bases, the cleavage product migrates as a band of two bases slower mobility than the substrate band. (B) Ligation activity of Cropo4, Cropo6, or Cropo8. The ligation reaction is schematically depicted in the right panel. The substrate, OL52 + OL50/OL51, used for assaying ligation allows covalent attachment of the enzyme to the 3′-end of the 5′-radiolabeled scissile strand (OL52) by cleaving off a dinucleotide. The dinucleotide disassociates from OL51 and allows ligation to OL50. A 53-mer ligation product is generated during the reaction. OL52 + OL50/OL51 was incubated with HT (lane 1), Cropo6 (lane 2), or Cropo8 (lane 3) at 37 °C. Reactions were continued for 120 min and terminated by the addition of SDS. Reaction products were analyzed by electrophoresis on a 12% denaturing polyacrylamide gel and autoradiography. Key: *, 5′-radiolabeling with γ-^{32}P; ●, 5′-cold phosphorylation; S, substrate; C, cleavage product; L, ligation product.

that the band corresponding to the cleavage product (denoted C) is retarded in the gel relative to the band corresponding to the substrate (denoted S) due to the covalent attachment of a trypsin resistant peptide having a gel electrophoretic mobility equivalent to approximately five nucleotides. Note also that, in the gel, samples containing HT were spaced from samples containing the Cropos by empty lanes. This was due to the different treatment of the samples (necessitated by pronounced differences in cleavage activity) before analysis, requiring the loading of markedly different amounts of radioactive sample generated by incubation with HT or the Cropos, respectively. The different treatment of samples also means that the relative cleavage efficiencies cannot be

accurately measured due to the lack of an internal loading control since the majority of the cleavage substrate is lost in the water phase during the phenol extraction. However, a reproducible significantly lower amount of cleavage complexes (approximately 10−20%) was isolated from the phenol interphases after cleavage with Cropo6 or Cropo8 as compared to the amount of cleave products formed by HT (determined by quantitative scanning). This indicates that the cleavage activities of Cropo6 and Cropo8 are considerably reduced relative to the wild-type enzyme. In fact, the amount of cleavage complexes formed by Cropo6 or Cropo8 was too low to be detected if phenol extraction and interphase isolation were omitted (data not shown).

DNA ligation was assayed by incubating a 50-fold molar surplus of HT, Cropo6, or Cropo8 with radiolabeled OL52 + OL50/OL51 for 120 min at 37 °C. Following EtOH precipitation and trypsin digestion the reactions were analyzed in a 12% (w/v) denaturing polyacrylamide gel. Cleavage/ligation of the utilized substrate will result in the release of two nucleotides from the radiolabeled 37-mer scissile strand (OL52) which subsequently will be joined to the 18-mer ligation strand (OL50) resulting in a 53-mer radiolabeled ligation product. As evident from Figure 6B, left panel, HT cleaved approximately 80% of the DNA substrate, and about 60% of the generated cleavage complexes (denoted C) were converted into the ligation product (denoted L) (lane 1). Ligation product also appeared after incubation of the substrate with Cropo6 (lane 2) and Cropo8 (lane 3), albeit at a low efficiency, corresponding to approximately 10% of the DNA substrate. No activity was observed for Cropo4 (data not shown). In contrast to the wild-type enzyme, no cleavage product was visible after incubation of the substrate with Cropo6 or Cropo8, indicating ligation of close to 100% of the cleavage products and suggesting a shift of the cleavage−ligation equilibrium toward ligation.

DISCUSSION

In the present study we show that the extended linker domain of human topo I can be replaced by a short loop structure from the corresponding region of Cre recombinase and still retain catalytic activity, albeit at a reduced level. Replacing residues 631−717 of human topo I by residues 314−317 from Cre (Cropo4) abrogates detectable relaxation activity whereas replacement of the human topo I residues 631−717 by the Cre residues 312−317 (Cropo6) renders the enzyme relaxation competent, and hence the six residues from Cre define the minimal linker region accommodated by the structural framework of human topo I. A third chimeric enzyme including residues 312−319 of the Cre linker region (Cropo8) was also relaxation competent.

The DNA relaxation activity of Cropo6 and Cropo8 correlated with their ability to form covalent cleavage complexes when incubated with the synthetic suicide DNA substrate (OL19/OL27), suggesting the reaction to proceed via the normal type IB topoisomerase mechanism involving formation of a covalent phosphotyrosyl linkage between the catalytic tyrosine and the scissile phosphate. This suggests that the catalytic tyrosine is rendered in a cleavage-competent conformation in Cropo6 and Cropo8, which seems not to be the case in the relaxation-incompetent Cropo4 since this variant was inert in DNA cleavage, although the enzyme retains wild-type DNA binding capacity. Both Cropo6 and Cropo8, but not Cropo4, were further able to catalyze ligation of a 5′-OH DNA end to the cleavage complex. On a synthetic DNA substrate which allows DNA ligation (OL52 + OL50/OL51), cleavage complexes formed by Cropo6 or Cropo8 were readily converted to ligation products whereas only 60% of cleavage complexes formed by HT were converted to ligation products at similar reaction conditions. Although the kinetics of the reactions have not been addressed due to limitations posed by the low activity levels of the Cropo variants (necessitating incubation times in time course experiments longer than the time frame in which the enzymes retain full activity at 37 °C), this result suggests that the cleavage−ligation equilibrium of Cropo6 and Cropo8 is shifted toward ligation. Similar shifts of the cleavage−ligation equilibrium have been reported for several other variants of human topo I carrying point mutations (*32*), deletions (*18*), or interruptions in the linker domain (*33, 34*). Likewise, the insensitivity of Cropo6 and Cropo8 toward CPT in DNA relaxation is in concordance with the characteristics of other human topo I mutants altered in the linker domain, most probably resulting from the lack of functional control of the strand rotation step of catalysis as have previously been described for linker-deleted (or mutated) human topo I variants (*18, 32, 34, 35*).

Reflecting the considerable flexibility of their active sites, human topo I, vaccinia virus topo I, and Flp are all capable of employing various nucleophiles (e.g., the hydroxide anion) during the transesterification reactions that they mediate. Whereas human topo I and vaccinia virus topo I catalyze the nucleophilic attack of hydroxide on the phosphotyrosyl bond formed upon cleavage (*26, 27*), Flp accepts the hydroxide anion as the nucleophile both during cleavage and ligation (*24, 28–30*). In the present study it was found that Cropo4, Cropo6, or Cropo8 exhibited considerable DNA nicking activity on supercoiled plasmid in the presence of hydrogen peroxide. This implies that all three chimeras are capable of exploiting the hydroxide anion as the nucleophile in the cleavage and/or the ligation reaction. Since Cropo4 is unable to form covalent complexes with DNA, it is most likely that this variant utilized hydrogen peroxide as a nucleophilic substitute for the active site tyrosine in the cleavage step of catalysis. Hence, the ability of Cropo4 to catalyze hydrogen peroxide assisted DNA nicking suggests that this enzyme harbors the capacity to activate the scissile phosphodiester bond for nucleophilic attack. The lack of catalytic activity of Cropo4 in the absence of an exogenous nucleophile is, therefore, most likely the result of a displacement of the catalytic tyrosine into an inactive conformation. Cropo6 and Cropo8 may employ the exogenous nucleophile during reactions analogous to both cleavage and ligation, which cannot be distinguished in the applied assay. However, the fact that both Cropo6 and Cropo8 exhibited increased hydroxide nicking activity compared to HT, while showing a decreased activity in cleavage, suggests that the hydroxide anion is used as a nucleophile at least in the cleavage step of the Cropo6/Cropo8 reactions. The increased nicking activities of Cropo6 and Cropo8 may offer an explanation for their reduced cleavage/relaxation activities, which did not correlate with a reduced DNA binding affinity, since it suggests a slight displacement of the catalytic tyrosine from

its optimal position in the active site (although not as severe as the one anticipated in Cropo4). Such displacement would leave the catalytic pocket in a more open conformation than that of the wild-type enzyme and potentially allow increased access of the hydroxide anion.

Finally, it should be noted that the described domain exchange replaces the essential active site residue His-632 of human topo I with the corresponding Trp-315 of Cre. Biochemical and structural data suggest these residues to play analogous roles during catalysis (*9, 11, 36, 37*), and it may be inferred from our study that the function of His-632 (human topo I sequence) can be complemented by a tryptophan at least in Cropo6 and Cropo8. This is in contrast to a previous report demonstrating that replacement of His-632 of human topo I with tryptophan devastates catalytic activity (*36*) and may reflect a slightly altered structural framework of the Cropo catalytic pocket compared to the wild-type enzyme. The replacement of histidine with tryptophan may explain the reduced activity of the two cleavage-competent Cropo variants. However, we find this unlikely since both Cropo variants exhibited an increased activity in the hydroxide nicking assay. Mutations of Trp-330 in Flp (corresponding to His-632 in human topo I) which renders the enzyme inactive in cleavage also diminish the utilization of hydrogen peroxide (*38*), suggesting similar roles of the residue during the two types of reaction. Further investigations aiming to fully elucidate the functions of residue 632 in topo I are currently being performed.

ACKNOWLEDGMENT

We are grateful to Associate Professor Birgitta R. Knudsen for kindly providing the construct for expression of HT and for inspiring discussions during the preparation of the manuscript. We also thank Professor Makkuni Jayaram for providing Cre enzyme and substrates for the Cre assays.

SUPPORTING INFORMATION AVAILABLE

Results showing CPT sensitivity of Cre recombinase (Figure S1) and gel showing salt optimum for DNA relaxation by Cropo4 (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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BI800031K