A Functional Linker in Human Topoisomerase I Is Required for Maximum Sensitivity to Camptothecin in a DNA Relaxation Assay*

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Human topoisomerase I is composed of four major domains: the highly charged NH2-terminal region, the conserved core domain, the positively charged linker domain, and the highly conserved COOH-terminal domain. Near complete enzyme activity can be reconstituted by combining recombinant polypeptides that approximate the core and COOH-terminal domains, although DNA binding is reduced somewhat for the reconstituted enzyme (Stewart, L., Ireton, G. C., and Champoux, J. J. (1997) J. Mol. Biol. 269, 355–372). A reconstituted enzyme comprising the core domain plus a COOH-terminal fragment containing the complete linker region exhibits the same biochemical properties as a reconstituted enzyme lacking the linker altogether, and thus detachment of the linker from the core domain renders the linker non-functional. The rate of religation by the reconstituted enzyme is increased relative to the rates of the enzyme containing the linker indicating that in the intact enzyme the linker slows religation. Relaxation of plasmid DNA by full-length human topoisomerase I or a 70-kDa form of the enzyme that is missing only the non-essential NH2-terminal domain (topo70) is inhibited ~16-fold by the anticancer compound, camptothecin, whereas the reconstituted enzyme lacking the linker altogether is nearly resistant to the inhibitory effects of the drug despite similar affinities for the drug by the two forms of the enzyme. Based on these results and in light of the crystal structure of human topoisomerase I, we propose that the linker plays a role in hindering supercoil relaxation during the normal relaxation reaction and that camptothecin inhibition of DNA relaxation depends on a direct effect of the drug on DNA rotation that is also dependent on the linker.

Eukaryotic topoisomerase I is a monomeric enzyme that promotes changes in DNA topology by introducing a transient break in the phosphodiester bond of one strand in duplex DNA (reviewed in Refs. 1 and 2). The transient break allows helical tension to be released by the unwinding of positively supercoiled DNA or the rewinding of negatively supercoiled DNA. Phosphodiester bond energy is preserved during catalysis through the formation of a covalent link between the active site tyrosine and the 3′ end of the broken strand. Strand closure follows the reverse path with reformation of the phosphodiester linkage and release of the enzyme. No energy co-factor or metalation is required for topoisomerase I activity.

Topoisomerase I is of significant medical interest as the sole intracellular target of camptothecin (CPT)1 (3–8), a plant alkaloid that rapidly blocks both DNA and RNA synthesis in treated cells (9–11). CPT and its derivatives have been shown to be effective in the treatment of a broad spectrum of human cancers (12–14). These compounds bind specifically and reversibly to the transient covalent enzyme-DNA complex (15, 16) and impede the religation step (17–19). Consequently, CPT enhances the yield of permanently trapped enzyme-DNA covalent complexes when in vitro reactions are terminated with a denaturant such as SDS (17). Numerous structure-activity studies have established a direct correlation between the ability of CPT and its derivatives to slow religation and their capacity to inhibit the relaxation of superhelical tension in DNA (15, 20, 21). Based on such studies, it is generally believed that the inhibitory effects of CPT on topoisomerase I relaxing activity are a direct consequence of the effect of the drug on religation.

The three-dimensional structure of an NH2-terminally deleted 70-kDa form of human topoisomerase I (topo70) (Fig. 1) bound to a high affinity duplex DNA substrate has recently been solved (22). This structural information, together with previous hydrodynamic measurements and limited proteolysis studies, has enabled us to precisely define the domain boundaries of human topoisomerase I (Fig. 1) (22–26). The highly charged NH2-terminal domain (Met1–Gly214) is largely disordered, contains four putative nuclear localization signals (27), is extremely sensitive to proteolysis, and can be eliminated with no effect on the in vitro activity of the enzyme (24, 28–30). The conserved core domain (Ile215–Ala635) can be divided into three subdomains. The first half of the core is composed of subdomains I and II, which form a “cap” structure that wraps half way around the DNA and has two positively charged “nose cone” helices that are positioned above, but do not contact the DNA in the region downstream of the site of covalent attachment (Fig. 2, black). The second half of the core domain, subdomain III (Fig. 2, red), is structurally related to the integrase family of tyrosine recombinases (23). This portion of the core domain is referred to here as the “catalytic” segment of the enzyme since it contains three essential active site residues, Arg188, Arg290, and His632 (22, 23). The COOH-terminal domain (residues Gln713 to Phe765, Fig. 2, green) forms a tight complex with core subdomain III and completes the active site

* This work was supported by Grant GM49156 from the National Institutes of Health (to J. J. C.) and by American Cancer Society Grant PF-3905 (to L. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CPT, camptothecin; topo70, NH2-terminal truncation of human topoisomerase I lacking the first 174 amino acids; topo58, COOH-terminal truncation of topo70 missing the last 106 amino acids; topo12, 108 amino acid COOH-terminal fragment of human topoisomerase I composed of amino acids 658–765; topo3, 53 amino acid COOH-terminal fragment of human topoisomerase I composed of amino acids 713–765; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol.
by providing the nucleophilic Tyr\textsuperscript{723} (6, 28). Together, the core and COOH-terminal domains wrap completely around the DNA making numerous contacts with phosphates in the 2\textsuperscript{5} to 1\textsuperscript{1} duplex region where the break site is defined to be between the 2\textsuperscript{1} and 1\textsuperscript{12} positions on the scissile strand. The linker domain comprising residues Pro\textsuperscript{636} to Lys\textsuperscript{712} connects the core and COOH-terminal domains (Fig. 2, orange). The linker assumes a protruding coiled-coil conformation that is stabilized by hydrophobic side chain interactions between conserved heptad repeating residues. Importantly, the conserved side chains of linker residues Lys\textsuperscript{650} and Arg\textsuperscript{708} form hydrogen bonds with phosphates downstream of the break site in the 1\textsuperscript{8} to 1\textsuperscript{10} duplex region of the DNA. The COOH-terminal fragments were generated by factor Xa digestion of a recombinant GST fusion proteins. Consequently, topol2 has 2 extra amino acids (Gly-Ile) at its NH\textsubscript{2} terminus, and topol6.3 has 3 extra residues (Gly-Ile-Pro) at its NH\textsubscript{2} terminus.

FIG. 1. The domain organization and structures of recombinant constructs. Human topoisomerase I is composed of four major domains: the highly charged NH\textsubscript{2}-terminal domain (Met\textsuperscript{1}–Gly\textsuperscript{214}), the conserved core domain (Ile\textsuperscript{215}–Arg\textsuperscript{635}), a positively charged linker region (Pro\textsuperscript{636}–Lys\textsuperscript{712}), and the highly conserved COOH-terminal domain (Gln\textsuperscript{713}–Phe\textsuperscript{765}). The primary structures of various recombinant proteins are as follows: topo70, a 70-kDa NH\textsubscript{2}-terminally truncated enzyme that starts with an engineered methionine immediately upstream of Lys\textsuperscript{175}; topol58, a 58-kDa core domain that has the same initiating methionine as topol70 but is terminated after residue Ala\textsuperscript{659}; topol2, the COOH-terminal 12 kDa of topoisomerase I encompassing residues Leu\textsuperscript{658}–Phe\textsuperscript{765}; and topol6.3, the COOH-terminal 6.3 kDa of topoisomerase I encompassing residues Gln\textsuperscript{713}–Phe\textsuperscript{765}. The COOH-terminal fragments were generated by factor Xa digestion of a recombinant GST fusion proteins. Consequently, topol2 has 2 extra amino acids (Gly-Ile) at its NH\textsubscript{2} terminus, and topol6.3 has 3 extra residues (Gly-Ile-Pro) at its NH\textsubscript{2} terminus.

[FIG. 2. The crystal structure of human topoisomerase I. A stereodiagram shows human topo70 bound non-covalently to DNA. Together, the core subdomains I and II comprise the “cap” region of human topoisomerase I (black), with helices \(a_5\) and \(a_6\) forming the “nose cone.” Core subdomain III is structurally homologous to the integrase family of tyrosine recombinases (red). The “linker” domain (orange) forms a coiled-coil structure that makes two contacts, mediated by residues Lys\textsuperscript{650} and Arg\textsuperscript{708} shown in cyan, with the duplex DNA located downstream of the topoisomerase I cleavage site. The positively charged side chains of nose cone residues, which face the DNA, are also shown in cyan. The non-cleaved strand of the DNA is shown in blue, while the scissile strand is shown in dark magenta upstream (~10 to –1) of the nicking site and in pink downstream (+1 to +12). The atoms of the active-site residues, Arg\textsuperscript{488}, Arg\textsuperscript{590}, His\textsuperscript{632}, and Tyr\textsuperscript{723} are rendered in cyan Van der Waals radii. The COOH-terminal domain (green) binds to core subdomain III.]
Lyso50 is part of the crystallized topo58/6.3-DNA complex, it is contained within a 24-amino acid region (residues Pro636–Ala655) that fails to adopt a stable conformation in the crystalline state. The loss of these two linker-DNA contacts provides a possible explanation for the ~20-fold reduced DNA affinity exhibited by the reconstituted enzyme. Evidence that the linker domain also contacts DNA in solution is supported by proteolysis studies, which show that the linker is ~10-fold more resistant to limited proteolysis when the enzyme (full-length or topo70) is bound non-covalently to duplex DNA (25).

In the present work we have extended our biochemical comparison of the intact and reconstituted enzymes and have found that CPT inhibits plasmid DNA relaxation by the reconstituted enzymes (topo58/12 and topo58/6.3) by less than 2-fold under standard assay conditions, whereas relaxation by topo70 is inhibited ~16-fold under the same conditions. We show that this effect is not the result of reduced binding of CPT to the reconstituted enzymes as compared with topo70. These results implicate the linker region of the enzyme in CPT inhibition of plasmid DNA relaxation.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Characterization**—The purification of topo70 and the preparation of the reconstituted enzymes (topo58/12 and topo58/6.3) (Fig. 1) were carried out as described previously (24, 26). The percentage of active reconstituted topoisomerase I molecules (topo58/12 or topo58/6.3) present in the final purified reconstituted enzyme preparations varied from one batch to the next. The reason for the inactivity of a subpopulation of the reconstituted molecules is not known, but it does not appear to be the consequence of dissociation of the core and COOH-terminal fragments since the gel filtration profile of the reconstituted enzyme invariably showed a single homogeneous protein species. The gel filtration procedure used to estimate the apparent molecular masses of topo58/12, topo58/6.3, and topo70 was carried out as described previously (24).

**Plasmid Relaxation Assays for Topoisomerase Activity**—Protein samples were serially diluted 2-fold in dilution buffer (10 mM Tris-hydrochloride, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml BSA) until the desired enzyme concentration was attained. The time-course assay used for the measurement of activity under various ionic conditions was initiated by mixing 12-μl of diluted enzyme with 228-μl of dilution buffer containing 25 ng/μl supercoiled plasmid substrate DNA. The buffer constituents common to all reactions were 10 mM Tris-hydrochloride, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.1 mg/ml BSA. The final KCl concentration was either 25, 150, or 300 mM. When included, CPT was at 50 μM and MgfCl2 was at 10 mM. The reactions were warmed to 37 °C and at various times 20-μl aliquots were stopped by the addition of 5 μl of Stop Dye (2.5% SDS, 25% Ficoll 400, 0.03% bromophenol blue, 0.03% xylene cyanol, 0.03% bromphenol blue), boiled, and analyzed by denaturing urea/polyacrylamide gel electrophoresis. The amount of breakage in the presence of CPT relative to the control sample without drug was determined by PhosphorImager analysis.

**Kinetics of Religation Using Oligonucleotide Substrates**—The CL14 oligonucleotide (see Fig. 8A) was radiolabeled at its 5′ end with [γ-32P]ATP and T4 polynucleotide kinase. The CP25 complementary strand was phosphorylated at its 5′ end with unlabeled ATP and polynucleotide kinase. The two strands were annealed at a 2-fold molar excess of CP25 over CL14 by heating to 95 °C for 1 min, followed by cooling to 25 °C in 60 min, and stored at 4 °C. The duplexes were analyzed by native polyacrylamide gel electrophoresis and autoradiography to ensure that the radiolabeled strand was fully duplexed.

Suicide cleavage was allowed to occur by incubating a 10-fold molar excess of CP25 oligonucleotide (0.2 μM) with the 5′-32P-labelled CL14/CP25 duplex (20 μM) for 60 min at 37 °C. The reaction was terminated by addition of 1 mM EDTA and 1 mM DTT. Under these conditions, suicide cleavage of 70–90% of the input DNA was routinely observed. Where indicated CPT was added from a stock diluted in MeSO to a final concentration of 50 μM (final MeSO concentration 1–5%). Religation reactions were initiated by adding a 200-fold excess of R11 oligonucleotide (see Fig. 8A) to the CL14/CP25 substrate reactions. Reactions were incubated at 37 °C, and at various time points 5-μl aliquots were removed and adjusted to 0.5% SDS. Samples were ethanol-precipitated and resuspended in 5 μl of 1 mg/ml trypsin and incubated at 37 °C for 30 min. The products were analyzed by denaturing urea/polyacrylamide gel electrophoresis as described above. Control experiments using a 2000-fold excess instead of a 200-fold excess of R11 oligonucleotide yielded identical rates of religation, showing that the rate of anneling of R11 was not rate-limiting for the religation reaction.

The rate of approach to equilibrium in the religation assay (θm) was measured and used to estimate the first order rate constant for religation (k2) as described by Stivers et al. (34). Briefly, the relative amounts of religation product and remaining covalent complex were determined by PhosphorImager analysis of the bands in the urea-polyacrylamide gel (see Fig. 8). The amount of complex remaining (% norm (% complex), (% complex), a – a) was plotted against time in minutes. From a semilog plot of these data, a value for θm was obtained, which was set equal to k2 + h. The plateau value for the percent cleavage at the end of the experiment was used to estimate the value of Kc religation rate equilibrium value or speed of relaxation reaction. For known reasons, the plateau values for topo58/12 in the absence of CPT did not reflect the expected 20-fold shift toward relaxation based on previous results (26). However, given the high rates for reliation in this case, the resultant uncertainty in the value of Kc had very little effect on the final calculated values for the relocation rates. Simultaneous solution of the above two equations yielded an estimate of the single-order rate
The Effect of CPT on Relaxation of DNA by Reconstituted Human Topoisomerase I—We examined the effect of 50 μM CPT on the ability of the intact (topo70) and reconstituted (topo58/12 or topo58/6.3) enzymes to mediate relaxation of supercoiled plasmid DNA under standard assay conditions containing 150 mM KCl. Under these conditions in the absence of CPT, the rate of relaxation of plasmid DNA by the same amount of reconstituted topo58/12 is only 2–4-fold less than the rate for topo70 (Fig. 3, compare panels A and C; note time is in seconds in panel A). Given that ~40% of the topo58/12 molecules are inactive (see “Experimental Procedures”), the reduction in activity for the active molecules is likely to be closer to 2-fold than 4-fold. However, in the presence of CPT it can be seen that the time to complete relaxation for topo70 is increased approximately 10–15-fold by CPT (from 1 min to 10–15 min; Fig. 3, compare panels A and B), whereas the drug has at most a 2-fold effect on the rate of relaxation by the reconstituted topo58/12 (Fig. 3, compare panels C and D). Since ionic strength has a large influence on enzyme activity (24), additional relaxation assays were carried out at three different KCl concentrations (25, 150, and 300 mM). The ratios of the activities in the presence of CPT to the activities in the absence of the drug under the various assay conditions are presented in Table I. Lowering the KCl concentration to 25 mM increased the inhibitory effects of CPT slightly for all three forms of the enzyme, but the magnitude of the inhibition remained much greater for topo70 as compared with either of the reconstituted forms of the enzyme. As shown previously (26), the reconstituted enzymes were inactive above 250 mM KCl. Notably, relaxation of the plasmid DNA by topo70 was nearly unaffected by CPT when the relaxation assay was performed at a high salt concentration (300 mM KCl), indicating that at the higher ionic strength the behavior of the intact enzyme is similar to that of the reconstituted enzymes under standard conditions of 150 mM KCl.

One possible explanation for the reduced effect of CPT on relaxation by topo58/12 and topo58/6.3 is that the reconstituted enzymes have a reduced affinity for CPT relative to the intact topo70. If so, one would predict that higher drug concentrations would be required to achieve the same high level of inhibition observed with the intact enzyme in a plasmid relaxation assay. To address this possibility, the concentration dependence of the inhibitory effects of CPT on DNA relaxation by the intact and reconstituted enzymes was determined. The results shown in Fig. 4 confirm that CPT inhibits plasmid relaxation by topo70 at all concentrations tested in the range from 2 to 200 μM, but for the reconstituted topo58/12, there was no significant increase in the inhibitory effects of CPT in the concentration range between 10 and 200 μM. This result suggests that differences in CPT binding affinity of the reconstituted enzyme versus topo70 cannot explain the differential effects of CPT on plasmid relaxation.

Another possibility to explain the greatly reduced inhibition of the reconstituted enzymes by CPT is that the reconstituted enzymes carry out cleavage and religation at sites different from the intact enzyme and that these sites are unaffected by CPT. However, this possibility is ruled out by our previous observation that topo58/12 and topo58/6.3 exhibit the same cleavage specificity as topo70 (26).

Gel Filtration Analyses of Topo70, Topo58/12, and Topo58/6.3—As shown above, reconstituted enzymes produced either by introducing a discontinuity in the linker domain (topo58/12) or by eliminating most of the linker altogether (topo58/6.3) exhibit a greatly reduced sensitivity to CPT in a plasmid relaxation assay. One possible explanation for the similarities between topo58/6.3 and topo58/12 is that a functional linker is
Reduced Camptothecin Sensitivity

FIG. 4. The concentration dependence of the inhibitory effects of CPT on topo70 and topo58/12. Enzyme assays were carried out at the indicated concentrations of CPT, and the -fold inhibition relative to the no-drug control is plotted as a function of the CPT concentration.

required for the inhibitory effects of CPT on DNA relaxation and that the discontinuity in the polypeptide chain at the beginning of the linker region in the case of topo58/12 disables the linker. To approach this possibility, we compared the gel filtration properties of topo58/12 with those of topo70 and topo58/6.3. As shown in Fig. 5, topo70 and topo58/6.3 chromatograph with apparent molecular masses that are inflated by 21 and 31 kDa, respectively. We previously argued, based on hydrodynamic properties and limited proteolysis studies, that the discrepancy for topo70 could be accounted for by an unstructured and highly extended region of ~40 amino acid residues (Lys175 to Gly214) at the NH2 terminus of the protein (24). That these residues are indeed disordered in both topo70 and topo58/6.3 is supported by the failure to detect any significant electron density for this region in three separate x-ray structure determinations (22, 23).

Topo58/12 chromatographed with an apparent molecular mass of ~134 or ~40 kDa larger than topo70 (Fig. 5). However, topo58/12 and topo70 only differ in total polypeptide composition by the presence of 4 extra amino acids at the NH2 terminus of topo12 (two from the vector and two that are duplicates of residues found at the COOH terminus of topo58) (26). The large apparent molecular mass of topo58/12 is not due to dimerization of the protein, since glycerol gradient sedimentation and native gel electrophoresis experiments demonstrate that topo58/12 is monomeric (data to be presented elsewhere). The presence of the active site tyrosine residue at position 723 and the similar activities of topo58/6.3 and topo58/12 in enzyme assays (26) suggests that the COOH-terminal region common to both (Gln713 to Phe766) is properly folded in topo58/12. Thus, it appears that the larger apparent size of topo58/12 that results from uncoupling the linker domain from the core domain is due the improper folding or reorientation of the linker region relative to the remainder of the protein (see “Discussion”).

Protection of the Linker Domain from Proteolysis by DNA—We have previously shown that the linker domain is protected from subtilisin digestion by a factor of ~10 when topo70 is bound non-covalently to DNA (25). The resistance to proteolysis in the presence of DNA can be readily explained by the observed linker-DNA contacts in the crystal structure of topo70 bound non-covalently to DNA (22). Given the above results implicating the linker in the sensitivity of the enzyme to CPT in a DNA relaxation assay, we were interested in the fate of linker-DNA interactions after the covalent complex had been formed. However, the only crystal structure of the enzyme in covalent complex with DNA is that of the reconstituted enzyme, topo58/6.3, which is missing the linker (23). Thus, in order to address this issue, we have examined the sensitivity of the linker region to limited proteolysis when topo70 is bound covalently to DNA. In this experiment, topo70 was allowed to react with a 1.2-fold molar excess of a high affinity duplex oligonucleotide substrate (CLts-CP) that has a 5’-bridging phosphorothioate bond at the site of topoisomerase I cleavage (Fig. 6A) (31). The resulting covalent complexes were subjected to digestion with 2-fold increasing concentrations of subtilisin (Fig. 6B, lanes 16–24). For comparison, equal quantities of free enzyme (no DNA present) were subjected to digestion under identical conditions (Fig. 6B, lanes 4–12).

A comparison of the digestion pattern of the free topo70 relative to that of the covalent topo70-DNA complex revealed that ~8-fold more subtilisin is required to eliminate all traces of the starting covalent complex versus the free enzyme (Fig. 6B, compare lane 24 to lane 9). In both cases, topo70 was cleaved within the linker region to produce a ~58-kDa core fragment that had the same gel mobility as the recombinant topo58 (Fig. 6B, compare lanes 12 and 13, and lanes 24 and 25). This 8-fold resistance to proteolysis is similar to that observed for topo70 bound non-covalently to DNA (25), and indicates that the linker domain likely remains associated with the downstream segment of DNA after formation of the covalent complex. At the lowest concentration of subtilisin a ~12-kDa fragment (Fig. 6B, lane 4) was produced that corresponds to cleavage at a protease-sensitive site near the boundary between the core domain and the linker. At the higher protease concentrations, further cleavages removed a portion of the linker to yield first a mixture of ~7–8-kDa fragments (Fig. 6B, lanes 6–8) and finally a protease-resistant COOH-terminal fragment that co-migrated with topo6.3 (Fig. 6B, compare lanes 12 and 13) and corresponds to the globular COOH-terminal domain (22, 23, 26). The absence of these fragments and presence instead of a ~12-kDa fragment at the higher subtilisin concentrations in the proteolysis series of the covalent complex (Fig. 6B, lanes 21–24) is best explained by a decrease in mobility of the proteolytic fragments by the covalently attached DNA oligonucleotide.

The Linker Domain of Topo58/12 Is Protease-hypersensitive—To gain further insight into the structure of the linker in

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2 G. C. Ireton, L. H. Parker, L. Stewart, and J. J. Champoux, manuscript in preparation.
topo58/12, we compared the limited subtilisin digestion pattern of free topo58/12 with that of topo70. This analysis was performed exactly as described above. When free topo58/12 was digested with the lowest concentration of subtilisin, the topo12 component was cleaved into a mixture of 7–8-kDa polypeptides (Fig. 6C, lane 4) whose gel banding pattern is almost identical to the 7–8-kDa digestion products produced when free topo70 was digested with a 32-fold higher concentration of protease (Fig. 6B, lane 9). The 7–8-kDa digestion products are the result of proteolytic removal of the linker region from topo58/12, we compared the limited subtilisin digestion pattern of free topo58/12 with that of topo70. This analysis was performed exactly as described above. When free topo58/12 was digested with the lowest concentration of subtilisin, the topo12 component was cleaved into a mixture of 7–8-kDa polypeptides (Fig. 6C, lane 4) whose gel banding pattern is almost identical to the 7–8-kDa digestion products produced when free topo70 was digested with a 32-fold higher concentration of protease (Fig. 6B, lane 9). The 7–8-kDa digestion products are the result of proteolytic removal of the linker region from...
topo12. With increasing subtilisin concentrations, the ∼7–8-kDa fragments were collapsed into a ∼6.3-kDa fragment with the same mobility as the recombinant topo6.3 (Fig. 6C, compare lanes 10–12 with lane 13). The finding that the linker region of topo58/12 is ∼32-fold more sensitive to proteolysis than the corresponding region of topo70 suggests further that this region in the reconstituted enzyme is unfolded or more exposed as compared with the intact enzyme.

Although the linker region of free topo58/12 is highly sensitive to proteolysis, we wondered whether it could be protected from proteolysis if the reconstituted enzyme were bound either non-covalently or covalently to DNA. To examine this possibility, we reacted topo58/12 with a 1.2-fold m excess of the suicide duplex substrate and subjected the material to digestion with 2-fold increasing concentrations of subtilisin as described above. (Fig. 6C, lanes 16–24). We chose to use a batch of topo58/12 in which approximately 40% of the enzyme molecules were inactive (see “Experimental Procedures”). This result was confirmed by SDS-PAGE analysis of topo58/12 before and after incubation with the suicide substrate, which revealed that ∼60% of the molecules had cleaved the suicide substrate producing an ∼18-kDa covalent oligonucleotide-topo12 complex, which migrated more slowly than the remaining 40% of free topo12 molecules (Fig. 6C, compare lanes 14 and 15). Although the reason for inactivity of 40% of the topo58/12 molecules is not fully understood, the gel filtration analyses demonstrated that the topo58/12 material is composed of a single macromolecular complex (Fig. 5), indicating that the inactivity of a subpopulation of molecules is not the result of disassembly of the topo58/12 complex or of extensive denaturation of a subpopulation of the molecules. Rather, it is more likely that the inactive molecules are the result of some unidentified chemical modification to either topo58 or topo12. Accordingly, we assume that the inactive topo58/12 molecules are still capable of binding DNA albeit non-covalently (see below).

These results indicate that the proteolytic sensitivity of the linker region of non-covalent and covalent topo58/12-DNA complexes are equal to each other and to that observed for the free topo58/12 (Fig. 6C, compare lanes 15 and 16 with lanes 3 and 4). In all cases, the linker region is cleaved at the lowest protease concentration to produce a doublet of COOH-terminal fragments of ∼7–8 kDa for the uncleaved topo12, or oligo-COOH-terminal fragments of ∼12–14 kDa for the covalent oligo-topo12 complex. Thus, while the proteolysis of the linker region of topo70 is inhibited 8–10-fold by non-covalent or covalent binding to duplex DNA, the linker region of the reconstituted topo58/12 remains sensitive to proteolysis at all times. These results provide further evidence that the linker region of the reconstituted topo58/12 is unfolded or exposed relative to the native intact enzyme. Taken together, these findings suggest that the discontinuity between the core and the linker regions of topo58/12 render the linker nonfunctional and thus explain why topo58/12 is so similar in its properties to topo58/6.3, which lacks the linker altogether. In the follow-up experiments described below, we chose to further characterize topo58/12 as representative of the two reconstituted enzymes.

Effects of CPT on the Cleavage-Religation Equilibrium for Topo70 and Topo58/12—Topo70 and topo58/12 were incubated with the 5’-32P-end-labeled duplex oligonucleotide shown at the top of Fig. 7 in the presence and absence of CPT. Equal amounts of topo70 (lanes 2–7) and topo58/12 (lanes 8–11) were incubated at the indicated KCl concentrations with the 5’-32P-end-labeled duplex oligonucleotide shown at the top of the figure. The arrow indicates the site of cleavage in the substrate. Where indicated, CPT was included in the reactions at 50 μM. The reactions were stopped with SDS, treated with trypsin and separated by urea/polyacrylamide gel electrophoresis followed by PhosphorImager analysis. The cleavage product (12-mer oligonucleotide containing residual topoisomerase I-peptide) migrated faster than the uncleaved CL25 oligonucleotide (lane 1).

8-fold, respectively (Fig. 7, compare lane 2 with lane 3 and lane 4 with lane 5). However, only low levels of cleavage were observed in the presence of CPT for topo58/12 at either salt concentration and cleavage in the absence of the drug was essentially at the limit of detectability (Fig. 7, lanes 8–11). Since these results were obtained at concentrations where all of the DNA is bound to the enzyme (26), the amount of cleavage is an approximate measure of the cleavage-religation equilibrium under the various conditions. The results in the absence of CPT are consistent with our previous findings indicating that the cleavage-religation equilibrium for the reconstituted enzyme is substantially shifted toward religation as compared with topo70 (Fig. 7, compare lanes 8 and 10 with lanes 2 and 4) (26). The low levels of cleavage observed in the presence of the drug for topo58/12 compared with the levels seen for topo70 (Fig. 7, compare lanes 9 and 11 with lanes 3 and 5) similarly reflect a shift in the cleavage-religation equilibrium toward religation. From these data, it was not possible to determine whether the CPT sensitivity of the reconstituted topo58/12 differed from that of topo70 due to the very low levels of cleavage in the absence of CPT (Fig. 7, lanes 8 and 10). However, the fact that cleavage is enhanced in the presence of the drug confirms that CPT does bind to the covalent complexes formed by topo58/12.

The cleavage observed for topo70 in the absence of CPT at 300 mM KCl was markedly reduced relative to that observed at 150 mM salt (Fig. 7, lanes 4–7), due in part to a reduction in DNA binding at the higher salt concentration (24). In several experiments similar to the one shown in Fig. 7, it appeared that...
the enhancement of cleavage by CPT for topo70 at 300 mM was reduced relative to the lower salt concentrations (compare lanes 6 and 7), but the low level of cleavage in the absence of the drug precluded a quantitative estimate of the effect (see below).

Effects of CPT on the Rates of Religation—To directly address the extent to which CPT affects religation by topo70 and topo58/12, religation rates for the two forms of the enzyme were measured in the absence and presence of the drug. A 5′-32P-end-labeled 14-mer oligonucleotide was annealed to a 25-mer oligonucleotide to generate the suicide cleavage substrate diagrammed in Fig. 8A. After allowing suicide cleavage to proceed to completion, a high concentration of a downstream complementary oligonucleotide was added and religation followed as a function of time by sequencing gel electrophoresis. Control experiments showed that annealing of the downstream oligonucleotide was not rate-limiting for the religation reaction. A typical experiment for topo70 at 150 mM KCl in the absence and presence of CPT is shown in Fig. 8B. The radioactivity in the religation product was quantified, and the results were plotted in Fig. 9A. The first order rate constant for religation was determined from these data (34), which gave a half-life for reclosure ($t_{1/2}$) of 4.6 min in the absence of CPT and 18.5 min in the presence of the drug (Table II). Thus, under these conditions, CPT slows closure for topo70 by a factor of 4. A similar experiment for topo58/12 yielded the results shown in Fig. 9B. For the reconstituted enzyme in the absence of CPT, more than half of the DNA is religated prior to the first time point (6 s) precluding an accurate determination of the religation $t_{1/2}$ in this case. Nevertheless, based on the results from several experiments, we estimate that CPT also decreased the initial rate of religation by topo58/12 by approximately a factor of 4 (Table II).

Given the lack of an effect of CPT on the rate of plasmid relaxation by topo70 at 300 mM salt (Table I), it was of interest to extend the religation kinetics to include measurements of the effect of CPT on the rate of religation by topo70 in the high salt condition. As can be seen in Table II, CPT had very little effect on the rate of religation by the wild type enzyme at 300 mM KCl. At 500 mM KCl the rate of religation in the presence of the drug was identical to the rate in the no drug control (data not shown). Notably, the rate of religation in the absence of the drug...
at 300 mM salt ($t_{1/2}$ of 1 min) is intermediate between the values observed for topo70 and topo58/12 at 150 mM KCl (Table II).

**DISCUSSION**

The evidence presented here indicates that the linker domain in the enzyme reconstituted by mixing the core (topo58) and COOH-terminal fragment (topo12) assumes a different conformation from that found in the intact enzyme (22). Topo58/12 chromatographs in a gel filtration analysis with an apparent molecular mass that is almost 50% larger than the apparent molecular mass observed for topo70 (134 kDa versus 91 kDa), despite the fact that the two proteins differ in amino acid composition only by the presence of 4 extra amino acids in topo58/12. Furthermore, although the intact linker region of topo70 is protected from limited proteolysis in both noncovalent (26) and covalent complexes with DNA (this work), the linker region in topo58/12 is as sensitive to proteolysis in the presence of DNA as in its absence. The most likely explanation for the difference in gel filtration properties and the sensitivity of the linker to proteolysis in the presence of DNA is that the linker domain is improperly folded or oriented differently relative to the remainder of the protein in topo58/12 as compared with topo70 (22). One possibility is that the detached linker in topo58/12 retains its coiled-coiled state (22), but swings further away from the body of the protein to a position which may even be perpendicular to the axis of the DNA (see Fig. 2). Such a re-orientation could dramatically increase the asymmetry of the molecule to affect its gel filtration properties and at the same time result in an increased accessibility to proteases both in the presence and absence of bound DNA.

Both of the reconstituted forms of human topoisomerase I studied here, topo58/12 and topo58/6.3, are less sensitive to CPT in a plasmid relaxation assay than is the wild type enzyme as exemplified by topo70. We propose that the lack of a functional linker accounts for the reduced sensitivity to CPT in both cases. While topo58/6.3 lacks the linker region altogether, topo58/12 appears to have a misfolded nonfunctional linker.

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**TABLE II**

**Rates of religation ($t_{1/2}$, min)**

| Enzyme       | 150 mM KCl $-$CPT | 150 mM KCl +CPT | 300 mM KCl $-$CPT | 300 mM KCl +CPT |
|--------------|-------------------|-----------------|-------------------|-----------------|
| Topo70       | 4.6               | 18.5            | 0.9               | 1.5             |
| Topo58/12    | ≤0.08             | 0.33            | ND               | ND              |

*ND, not determined.*

at 300 mM salt ($t_{1/2}$ of ~1 min) is intermediate between the values observed for topo70 and topo58/12 at 150 mM KCl (Table II).
resulting from the discontinuity in the polypeptide chain at the core-linker boundary. We chose to make more extensively characterize topo58/12 with respect to other parameters affected by CPT since it differs the least from topo70 in total amino acid content.

We previously showed that the lack of a linker region in the reconstituted enzymes shifts the cleavage-religation equilibrium toward religation by a factor of about 20 (26). Since the cleavage-religation equilibrium \( K_{\text{cl,rel}} \) is equal to \( k_{\text{rel}} / k_{\text{cl}} \) (34), such a shift could result from either an increase in the rate of religation or a decrease in the rate of cleavage. Since \( k_{\text{cl}} \) is inversely proportional to \( t_{1/2} \) for the religation reaction, the data in Table II allow us to estimate that there is an increase in \( k_{\text{rel}} \) by a factor of about 60 \((t_{1/2} \text{ of 4.6 min versus } -0.08 \text{ min})\) for the reconstituted topo58/12 relative to topo70. Thus, it appears that we can account for the entire shift in the cleavage-religation equilibrium upon loss of a functional linker by an increase in the rate of religation. If there is an effect on \( k_{\text{cl}} \), it would appear to be a modest one. Therefore, the presence of the linker in the intact enzyme under normal circumstances acts to slow religation. Since this effect is observed with oligonucleotide substrates where there is no supercoiling to drive strand rotation, the reduction in \( k_{\text{cl}} \) in the intact enzyme as compared with the reconstituted enzymes must result from some feature of the enzyme-DNA complex not related to the effects of the linker on DNA relaxation described below. Whether this effect acts directly at the level of the active site geometry to alter the chemistry of catalysis, or is mediated through DNA contacts by the linker downstream of the break site (see Fig. 2) (22) to distort the DNA such that reclosure is slowed remains unknown. The only crystal structure of human topoisomerase I in a covalent complex with DNA involves the reconstituted form of the enzyme missing the linker altogether (topo58/6.3) (23). Consistent with the above prediction, the 5’ hydroxyl of the broken strand is perfectly positioned for reclosure in this complex lacking the linker. At present, a crystal structure of the covalent complex with the linker present is not yet available to provide structural insights into how the linker slows the religation phase of the reaction. A corollary to the above considerations is that the rate-limiting step for religation by the linker-containing enzyme occurs at some stage prior to the actual religation chemistry as originally suggested by Stivers et al. (35).

A number of CPT-resistant forms of human and yeast topoisomerase I have been identified that involve single amino acid changes at a variety of sites within the protein (36). The most likely explanation for the loss of sensitivity to CPT in these cases is that a structural alteration in the protein results in a reduced affinity of the enzyme-DNA covalent complex for the drug. Since CPT slows religation for topo58/12 to the same extent as for topo70 (4-fold in both cases, Table II), the drug still binds to the covalent intermediates formed by the reconstituted enzyme. Furthermore, since the magnitude of the effects of CPT on DNA relaxation by topo58/12 cannot be increased by higher drug concentrations (Fig. 4), a reduced affinity for the drug is an unlikely explanation for the low degree of inhibition in plasmid relaxation. We therefore propose that the basis for the reduced sensitivity to CPT of topo58/12 and topo58/6.3 in the plasmid relaxation assay is likely to be found in some step beyond the binding of the drug to the enzyme-DNA covalent complex. Previous work has established a correlation between the ability of CPT to slow religation and its ability to inhibit DNA relaxation (15, 20, 21). The greatly reduced effect of CPT on relaxation by the reconstituted enzymes despite an effect on the rate of religation suggests that these two properties can be separated by altering the structure of the enzyme.

To understand how CPT inhibits the relaxation reaction by the wild type topoisomerase and why CPT sensitivity is lost in the absence of a functional linker, it is useful to consider the relationship between the various kinetic parameters that determine the rate of DNA relaxation. At a molar excess of plasmid DNA over enzyme, the overall rate of relaxation is a function of (i) the cleavage rate \( k_{\text{cl}} \), (ii) the dissociation rate of enzyme from the product, and (iii) the average number of supercoils that are relaxed per cleavage event \( \Delta n \). To a first approximation, we argue above that the cleavage rates for the two forms of the enzyme are equal, thus eliminating \( k_{\text{cl}} \) as a major determinant of the differential relaxation rates in the absence and presence of CPT. For topo70, the lack of topoisomerase intermediates during the course of the relaxation reaction (see Fig. 3, panels A and B) indicates that the enzyme completely relaxes a substrate in a processive fashion before dissociating and reassociating with another DNA molecule. It has been shown previously that the intact enzyme binds preferentially to supercoiled DNA over the relaxed product (37) and thus once the DNA is relaxed the enzyme readily redistributes to an unreacted substrate molecule. Furthermore, under the conditions of the relaxation assay, the concentrations of the enzyme and substrate are well above the dissociation constant and thus once dissociation does occur, rebinding is rapid. Thus, for topo70, we can assume that all of the enzyme molecules are bound to the DNA. However, under these conditions, the rate-limiting step in the multiple turnover relaxation reaction is likely to be the rate of release of the enzyme from the relaxed product as predicted by Stivers et al. (34). The situation with the reconstituted enzyme is somewhat different since partially relaxed topoisomerase are visible during the course of the relaxation reaction (see Fig. 3, panels C and D). Thus, in this case and consistent with the reduced affinity of the reconstituted enzyme for DNA (26), the reaction is distributive with the enzyme dissociating from partially relaxed molecules during the reaction. Finally, once cleavage of a supercoiled DNA has occurred, the DNA can either be reclosed without any relaxation, or supercoils can be relaxed before religation with a rate equal to \( k_{\text{rel}} \) (number of supercoils relaxed per second) (38). The ratio of the rate constants for these two competing reactions determines the number of supercoils that are removed each time the enzyme cleaves the DNA (i.e. \( \Delta n = k_{\text{sup}} / k_{\text{cl}} \)) (38). Although the ratio of these two parameters has not been measured for human topoisomerase I, the value of \( k_{\text{sup}} / k_{\text{cl}} \) for the vaccinia enzyme has been determined to be approximately 5, i.e. 5 supercoils are relaxed on the average for each nicking-closing cycle (38).

Based on these considerations, how can we account for the approximately 2-fold slower plasmid DNA relaxation rate for topo58/12 as compared with topo70 despite the very large increase in the rate of religation for the reconstituted enzyme? One factor that likely leads to the slightly slower rate is the reduced affinity of the reconstituted enzyme for the DNA so that, unlike topo70, not all of the enzyme molecules are bound to the DNA at any given instant. However, given that \( k_{\text{rel}} \) for topo58/12 is \( \sim 60 \) times greater than \( k_{\text{rel}} \) for topo70, if we assume equal rates of supercoil release by the two forms of the enzyme \( i.e. k_{\text{sup}} \text{(topo70)} = k_{\text{sup}} \text{(topo58/12)} \) then the number of supercoils relaxed per nicking-closing cycle \( \Delta n = k_{\text{sup}} / k_{\text{cl}} \) should be 60 times fewer for the reconstituted enzyme as compared with topo70. Since the number of supercoils released per nicking event is likely to be a major determinant of the overall rate of relaxation, it seems unlikely that a 60-fold reduction in the value of \( \Delta n \) would lead to less than a 2-fold reduction in the relaxation rate, especially since a reduction of this magnitude would mean that many nicking-closing cycles would likely be nonproductive with respect to DNA relaxation. Thus, it
seems probable that in the absence of the linker, the rate of supercoil relaxation is increased relative to the rate for topo70 (i.e. $k_{\text{sup}}\text{topo58/12} > k_{\text{sup}}\text{topo70}$) with a net effect of partially offsetting the large increase in $k_r$. Based on the crystal structure of human topoisomerase I in complex with DNA, we previously proposed that DNA rotation after cleavage is at least partially hindered by contacts between the rotating DNA and the bottom and top surfaces of the cap and linker regions, respectively (22) (called controlled rotation). If the loss of the linker does indeed lead to an increase in $k_{\text{sup}}$ then the presence of the linker in topo70 must decrease the rate at which supercoils are dissipated during DNA relaxation, a conclusion which directly supports the controlled rotation model.

The lack of a tight gripping interaction of the protein on the DNA downstream from the cleavage site in the crystal structure of the enzyme-DNA complex strongly favors some type of rotation model over a strict enzyme-bridging model for DNA relaxation (22). Moreover, it is well established that a major effect of CPT on the topoisomerase I-DNA covalent complex is relaxation (22). Moreover, it is well established that a major rotation model over a strict enzyme-bridging model for DNA relaxation (22). Moreover, it is well established that a major rotation model over a strict enzyme-bridging model for DNA relaxation.

Thus, in the absence of a functional linker in topo58/12 and in the absence of a functional linker (less than a 2-fold inhibition compared with topo70/6.3, the only remaining effect of the drug is to slow DNA relaxation). This hypothesis provides an explanation for the inhibition of topo70 in the plasmid relaxation assay by CPT despite the fact that the drug decreases the rate of religation.

How can we account for the greatly reduced effects of CPT on plasmid relaxation by the reconstituted enzymes lacking a functional linker (less than a 2-fold inhibition compared with topo70/6.3, the only remaining effect of the drug is to slow DNA relaxation). This hypothesis provides an explanation for the inhibition of topo70 in the plasmid relaxation assay by CPT despite the fact that the drug decreases the rate of religation.

How can we account for the greatly reduced effects of CPT on plasmid relaxation by the reconstituted enzymes lacking a functional linker (less than a 2-fold inhibition compared with topo70/6.3, the only remaining effect of the drug is to slow DNA relaxation).

Reduced Camptothecin Sensitivity

Acknowledgments—We thank Sharon Schultz, Zheng Yang, and Heidrun Interalh for helpful comments during preparation of the manuscript.

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