DNA relaxation by human topoisomerase I occurs in the closed clamp conformation of the protein

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In cocrystal structures of human topoisomerase I and DNA, the enzyme is tightly clamped around the DNA helix. After cleavage and covalent attachment of the enzyme to the 3' end at the nick, DNA relaxation requires rotation of the DNA helix downstream of the cleavage site. Models based on the cocrystal structure reveal that there is insufficient space in the protein for such DNA rotation without some deformation of the cap and linker regions of the enzyme. Alternatively, it is conceivable that the protein clamp opens to facilitate the rotation process. To distinguish between these two possibilities, we engineered two cysteines into the opposing loops of the "lips" region of the enzyme, which allowed us to lock the protein via a disulfide crosslink in the closed conformation around the DNA. Importantly, the rate of DNA relaxation when the enzyme was locked on the DNA was comparable to that observed in the absence of the disulfide crosslink. These results indicate that DNA relaxation likely proceeds without extensive opening of the enzyme clamp.

DNA topoisomerases are ubiquitous and essential enzymes that solve the topological problems accompanying key nuclear processes such as DNA replication, transcription, repair, and chromatin assembly by introducing temporary single- or double-strand breaks in the DNA (1–4). In addition, these enzymes fine-tune the steady-state level of DNA supercoiling to facilitate protein interactions with DNA and to prevent excessive supercoiling that is deleterious. There are two fundamental types of topoisomerases, which differ in both mechanism and cellular function (1). Type II enzymes are dimeric, promote the passage of one region of duplex DNA through a double-stranded break in the same or a different molecule, and are primarily dedicated to such processes as DNA supercoiling and chromosome segregation. Type I enzymes are monomeric and transiently break one strand of the duplex DNA, allowing for adjustments in helical winding. These enzymes are primarily responsible for removing torsional stress generated by processes that leave the DNA overwound or underwound. The type I topoisomerases have been further divided into two subfamilies based on sequence comparisons and reaction mechanisms (2). The type IA subfamily is characterized by covalent attachment of the enzyme to the 5' end of the broken strand in the nicked intermediate, whereas all members of the type IB subfamily attach to the 3' end of the broken strand. The prototype of the type IB subfamily, human topoisomerase I, catalyzes changes in the superhelical state of duplex DNA by transiently breaking one strand of the DNA to allow rotation of one region of the duplex relative to another region (5). Strand cleavage is achieved by the nucleophilic attack of the active site tyrosine on a DNA phosphodiester bond. The resulting formation of a phosphodiester bond between the tyrosine and the 3' end of the cleaved strand enables the enzyme to reseat the DNA by simple reversal of the cleavage reaction (1).

Human topoisomerase I is a 765-aa (91-kDa) enzyme that catalyzes the relaxation of both negative and positive supercoils in a reaction that does not depend on an energy-rich cofactor or divalent cations (1). Based on limited proteolysis studies and the crystal structure (6, 7), the protein can be divided into four discrete domains. An N-terminal domain comprising approxi-

mately the first quarter of the protein is highly charged and poorly conserved. This region of the protein is dispensable for enzymatic activity because a truncated form of the protein missing the first 174 aa (topo70) displays the same DNA relaxation activity as the full length protein *in vitro* (7). The remainder of the protein consists of the highly conserved core domain (54 kDa), the conserved C-terminal domain (8 kDa), which contains the nucleophilic tyrosine at position 723, and the poorly conserved and positively charged linker region (5 kDa) that connects the C-terminal domain to the core. In addition to tyrosine 723 located in the C-terminal domain, the active site is composed of residues found in the core domain of the enzyme.

Based on the crystal structure, topoisomerase I is a bi-lobed protein that clamps completely around duplex DNA through protein–DNA phosphate interactions (6). The core domain of the protein can be further divided into subdomains I, II, and III. One lobe of the protein, termed the cap region, consists of core subdomains I and II and sits on top of the duplex as shown in Fig. 1A. It is composed of mixed α and β secondary structural elements and contains two unique nose-cone helices ($\alpha 5$ and $\alpha 6$) that extend 25 Å from the body of the molecule (8). The second lobe (core subdomain III, the linker, and the C-terminal domain) sits below the DNA, is composed of an all α -helical structure except for one three-stranded β sheet, and contains the catalytic residues implicated in the strand cleavage and religation reactions (Fig. 1A) (8). In the closed clamp configuration found in the cocrystal structure, the two lobes are covalently joined through a continuous α -helical chain ($\alpha 8$) on one side of the DNA molecule and contact each other in the "lips" region on the opposite side of the DNA through the formation of a salt bridge between loops that extend from each of the lobes (Fig. 1C) (6).

Because all of the available crystal structures of human topoisomerase I contain bound DNA, little is known about the conformation of the DNA-free enzyme or the nature of the conformational changes that accompany DNA binding. However, examination of the cocrystal structure reveals that, in order for the DNA to dissociate from the enzyme, the two lobes must move apart to open the clamp, as modeled in Fig. 1D. Likewise, a similar open-clamp conformation must exist before DNA binding.

Despite considerable debate, the mechanism of DNA relaxation after formation of the covalent complex and before religation remains elusive. One of the conclusions drawn from the crystal structure was that the available space within the protein framework downstream of the cleavage site is insufficient to easily accommodate the rotation of the DNA helix required for DNA relaxation (5). It was noted that the DNA

Abbreviations: GSSG, oxidized glutathione; topo70, N-terminal truncation of human topoisomerase I missing the first 174 aa; topo70^{2xCys}, topo70 containing the H367C and A499C mutations.

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Fig. 1. Schematic showing the key structural features of human topoisomerase I. (A) The crystal structure of human topoisomerase I (residues 215–765) in complex with a 22-bp DNA (5, 6). Core subdomains I and II form the upper lobe or cap of the enzyme (magenta) and contain the nose cone helices. The lower lobe of the enzyme, shown in cyan, comprises subdomain III, the linker region, and the C-terminal domain. (B) The predicted structure for the disulfide bond formed between Cys-367 and Cys-499 in topo70^{2XCys}. The predicted distance between the C β atoms of the two cysteines is 4.6 Å. The two loops (Arg-362–Met-370 and Lys-493–Thr-501) follow the same color scheme as in A. (C) An end view of the cocrystal structure of human topoisomerase I looking down the axis of the DNA helix. The two opposing loops shown in B contact each other in a region referred to as “lips.” (D) Model of a hypothetical open clamp form of the protein (3).

proximal surfaces of the cap region and the linker contain 16 conserved positively charged side chains that could interact with the DNA and possibly hinder the rotation process. Attempts to model rotation within this cavity indicate that, if the protein were to remain in a closed clamp conformation as is found in the crystal structure, the rotation of the DNA would likely require at least a slight upwards shift of the cap and a downwards movement of the linker region (5). Alternatively, it has been proposed that the clamp opens after cleavage to accommodate the rotation of the DNA, as depicted in Fig. 1D (3). In this report, we describe experiments designed to discriminate between these two hypotheses.

Based on structure-modeling studies, a mutant form of human topoisomerase I was generated in which two proximal amino acids, each located in one of the opposing loops of the “lips” region, were changed to cysteines (topo70^{2XCys}). The two cysteine residues were predicted to be close enough to form a disulfide bond under oxidizing conditions (Fig. 1B) and thereby lock the enzyme in the closed clamp conformation around the DNA. Previously, a similar approach involving engineered disulfide bonds has been used successfully to probe the requirements for protein gate opening in the reactions catalyzed by both type IA and type II topoisomerases (9–11). Here, we show that topo70^{2XCys} forms a salt-stable complex with DNA under conditions that promote the formation of disulfide bonds and is released from the DNA in the presence of DTT. We have used such complexes to directly test whether clamp opening is required for DNA relaxation.

Materials and Methods

Expression and Purification of A499C/H367C Mutant Human Topoisomerase I. The plasmid pFASTBAC-topo70 codes for an N-terminal truncation of human topoisomerase I that begins at

residue 175 and retains full enzymatic activity when measured *in vitro*. The QuikChange site-directed mutagenesis kit from Stratagene was used to introduce the A499C and H367C mutations into pFASTBAC-topo70. The recombinant baculovirus expressing the resulting topo70^{2XCys} protein was generated with the Bac-to-Bac expression system according to the protocol provided by the manufacturer (Invitrogen), and the double mutant protein was purified from baculovirus-infected cells as described for WT topo70 (12). Briefly, the purification procedure involved fractionation on phosphocellulose, followed by Mono Q (5H/R) and Mono S (5H/R) chromatography by using the FPLC system from Amersham Pharmacia Biotech. The topo70^{2XCys} protein was eluted from the Mono S column and after dialysis into storage buffer (50% glycerol/10 mM Tris-HCl, pH 7.5/1 mM EDTA/5 mM DTT) was stored at –20°C under N₂ gas.

Disulfide Crosslinking of Topo70^{2XCys} on Plasmid DNA. The crosslinking reactions were initiated by the addition of 1 nmol of topo70^{2XCys} to crosslinking buffer (10 mM Tris-HCl, pH 7.5/30 mM KCl/1 mM EDTA/0.1 mg/ml of BSA) containing 10 pmols of supercoiled pRc/CMV plasmid DNA (Invitrogen) that had been chilled to 4°C (final volume 100 μ l). After incubation for 60 min at 4°C to allow complete relaxation of the plasmid DNA, either DTT (5 mM) or oxidized glutathione (GSSG; 15 mM) was added to the reaction. The incubations were continued for 16 h at 4°C, and the reactions were stopped by the addition of NaCl to 1 M before fractionation through a sucrose gradient. A parallel control reaction contained WT topo70 incubated with pRc/CMV DNA in the presence of GSSG.

Sucrose Gradient Sedimentation. Sucrose gradient sedimentation was performed by layering the products of the crosslinking reaction (125 μ l) onto a 3.8-ml linear 5–20% sucrose gradient containing 10 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM EDTA, and either 5 mM DTT or 10 mM GSSG, depending on which component had been present in the initial incubation. The gradients were centrifuged at 50,000 rpm in an SW60 rotor (Beckman) for 4.5 h at 20°C. Approximately 100- μ l fractions were collected from the bottom of the gradient tube through a small puncture, and the fractions were stored at 4°C. To locate the fractions containing DNA, 3- μ l samples of selected fractions were treated with proteinase K (100 μ g/ml) in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.1 mg/ml BSA (reaction buffer) for 2 h at 4°C. The reactions were stopped by the addition of 3 μ l of Stop Dye (25% Ficoll/0.03% bromophenol blue/0.03% xylene cyanol, 25 mM EDTA) and analyzed by electrophoresis in a 1% agarose gel at 6 volts/cm for 6 h at 4°C. After staining with 0.5 μ g/ml ethidium bromide, the electrophoresis was continued for an additional 2 h at the same voltage to separate the relaxed topoisomers from the nicked circles as described (13). The DNA bands were photographed with UV illumination.

Immunoblot Assays of Sucrose Gradient Fractions. Fifteen-microliter portions of selected sucrose gradient fractions were subjected to electrophoresis in a 10% SDS-polyacrylamide gel (14), and the proteins were electrophoretically transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) in 20% methanol/25 mM Tris–190 mM glycine at 200 mA for 2 h. After transfer, the membranes were blocked with 5% nonfat milk in Tris-buffered saline plus 0.05% Tween 20 (TBST) for 30 min at 23°C and then incubated for 12 h at 4°C with a 1:1,000 dilution of Scl-70 antiserum (Immunovision, Springdale, AR) in TBST plus 5% milk. After washing with TBST plus 5% milk, the membranes were incubated with a 1:20,000 dilution of affinity-purified horseradish peroxidase-conjugated goat anti-human IgG (GIBCO/BRL) at 23°C for 1 h in TBST plus 5% milk. After

additional washes with TBST, the wet membranes were incubated for 5 min at 23°C with the Supersignal West Pico Chemiluminescent Substrate detection system (Pierce). The light-emitting blots were sandwiched between two transparent sheets of plastic and exposed on a Storm 840 PhosphorImager (Molecular Dynamics).

Assays of Sucrose Gradient Fractions for Topoisomerase I Activity. To assay for the association of active topoisomerase I with pRc/CMV DNA in the sucrose gradient fractions, a 3- μ l sample of fraction 8 from each gradient was incubated with 0.15 μ g of an exogenous supercoiled plasmid DNA (pBluescript KS II⁺) in a relaxation reaction containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM DTT, 1 mM EDTA, and 0.1 mg/ml BSA (final volume 20 μ l). To verify that any relaxation activity observed depended on release of topo70^{2XCys} from the pRc/CMV DNA by reduction of the disulfide bond crosslink, an identical assay was carried out without DTT. The reactions were incubated for 30 min at 23°C and stopped by the addition of 2.2 μ l of 1 mg/ml proteinase K. After a further incubation for 30 min at the same temperature, the samples were adjusted to 1% SDS, and the products were analyzed by electrophoresis in 1% agarose gels (with and without 1.5 μ g/ml chloroquine) at 1.8 volts/cm for 14 h. The gels were stained with ethidium bromide and photographed by using a digital imager from Fotodyne (New Berlin, WI). Peak areas were determined by using SCION IMAGE FOR WINDOWS software (Scion, Frederick, MD).

To determine whether the topo70^{2XCys} enzyme associated with the pRc/CMV DNA after sucrose gradient sedimentation could cleave the endogenous DNA in the complexes, 3 μ l of fraction 8 was incubated in reaction buffer with or without camptothecin (10 μ M; final volume 20 μ l). A parallel reaction contained 10 mM DTT to release the enzyme from the DNA. After incubation at 37°C for 60 min, the reactions were stopped with 1% SDS to trap topoisomerase I-DNA covalent complexes. The samples were treated with proteinase K and analyzed by electrophoresis in a 1% agarose gel as described above. To determine the baseline level of nicked DNA, a parallel sample was treated with proteinase K before the addition of the SDS.

To assay for the ability of the disulfide crosslinked topo70^{2XCys} to relax the endogenous pRc/CMV DNA, fraction 8 from the sucrose gradient was incubated in reaction buffer (final volume 20 μ l) with or without added DTT (10 mM) at 23°C or 37°C for 60 min, and the reactions were stopped with proteinase K as described above. The starting distribution of topoisomers present in the sample stored at 4°C was determined by directly treating the sample with proteinase K for 2 h at 4°C, followed by agarose gel electrophoresis alongside the experimental samples incubated at the higher temperatures. For the time course analysis, the samples were preincubated for 60 min at 4°C in the presence of 10 mM DTT to release any crosslinked enzyme from the DNA before incubation for the indicated times at 37°C. Parallel incubations at 4°C were carried out in the absence of DTT. A portion of each reaction was stopped with proteinase K after the 60-min incubation at 4°C to establish the mobility pattern of the initial topoisomer distribution. For these experiments, chloroquine (0.3 μ g/ml) was included in the agarose gel to improve the resolution of the DNA topoisomers and the separation of the topoisomers from the nicked circles. Any relaxation of the endogenous DNA was detected by a shift of the topoisomer distribution from that characteristic for the conditions of storage (4°C) to that dictated by the temperature of incubation in the reactions without DTT.

Results and Discussion

Experimental Design. Modeling studies based on the crystal structure of human topoisomerase I complexed with a 22-mer duplex DNA (6) indicated that replacement of the amino acids at

Fig. 2. Assays of sucrose gradient fractions for plasmid DNA and topoisomerase I proteins. Plasmid pRc/CMV DNA was incubated at 4°C with topo70^{WT} under oxidizing conditions (GSSG, A), with topo70^{2XCys} under reducing conditions (DTT, B), or with topo70^{2XCys} under oxidizing conditions (GSSG, C). After centrifugation through sucrose gradients, aliquots of the indicated fractions were subjected to immunoblot assays for the presence of topoisomerase I protein. The lane marked C for A–C contained purified topo70, which provided a mobility standard for the immunoblot analysis. In D, additional aliquots of the same fractions were treated with proteinase K and analyzed by agarose gel electrophoresis to locate the pRc/CMV DNA in the gradient. The DNA profiles were very similar for all three gradients; only the fractions from the gradient for topo70^{2XCys} incubated with the DNA in the presence of GSSG are shown here. Lane C in D contained purified pRc/CMV DNA that had been relaxed by topo70 under the same conditions as used for the crosslinking experiments.

positions 367 and 499 with cysteines would place the C β atoms of the two cysteines 4.6 Å apart, a distance that is compatible with the formation of a disulfide bond under oxidizing conditions (Fig. 1B) (15, 16). The mutations H367C and A499C were introduced into topo70 by site-directed mutagenesis, and the specific activity of the resulting purified enzyme (topo70^{2XCys}), as measured by the plasmid relaxation assay, was reduced only 2- to 4-fold relative to WT topo70 (data not shown).

To promote the formation of a disulfide crosslink between the cysteines at positions 367 and 499, topo70^{2XCys} was incubated with pRc/CMV plasmid DNA in the presence of GSSG. In preliminary experiments, disulfide bond formation seemed to preferentially occur in the absence of bound DNA, and therefore, to increase the probability that a DNA molecule would contain disulfide crosslinked enzyme, we used a molar ratio of enzyme to DNA of 100. Under these conditions, the plasmid DNA became completely relaxed. A parallel control incubation contained DTT in place of GSSG to maintain the reduced state of the cysteines and prevent any disulfide crosslinking of the mutant enzyme on the DNA. To control for any effects of GSSG on the association of the enzyme with the DNA that were unrelated to the presence of the two added cysteine residues, a third sample containing WT topo70 in place of topo70^{2XCys} was incubated in the presence of GSSG.

To separate any protein–DNA complexes from free enzyme, the samples were sedimented through sucrose gradients containing 1 M NaCl, which should dissociate any enzyme not crosslinked to the DNA. Every third fraction of the sucrose gradients was assayed both for the presence of DNA by agarose gel electrophoresis and for any associated topoisomerase I protein by immunoblot analysis. Under these conditions, the pRc/CMV DNA sedimented to a position near the bottom of the gradients and was found in fractions 3, 6, and 9 in all three gradients (Fig. 2D, topo70^{2XCys}; data not shown for the other two gradients). As expected, the bulk of the topoisomerase I protein as detected by the immunoblot analysis was found near the top of all three gradients (Fig. 2A–C, fractions 21–27). However, a small fraction of topo70^{2XCys} was found to cosediment with the DNA when incubated with the DNA under oxidizing conditions (GSSG) (Fig. 2C, fractions 3, 6, and 9). No topoisomerase I protein cosedimented with the DNA in the

Fig. 3. Assay for the cosedimentation of topoisomerase I activity with pRc/CMV DNA. Supercoiled pBluescript KS II⁺ DNA (pKS II) was incubated under standard relaxation conditions with fraction 8 from each of the sucrose gradients shown in Fig. 2 (70^{WT}/GSSG, 70^{2XCys}/DTT, and 70^{2XCys}/GSSG) at 23°C in the absence of DTT (lanes 1, 3, and 5) to detect any nonspecific association of topoisomerase I with the sedimented pRc/CMV DNA. Similar incubations with exogenous pBluescript KS II⁺ DNA were carried out in the presence of DTT to detect any cosedimenting activity that could be released by reduction of disulfide bonds (lanes 2, 4, and 6). The reactions were stopped with proteinase K and analyzed by agarose gel electrophoresis in the absence (A, no chloroquine in gel) or presence (B, chloroquine in gel) of 1.5 µg/ml chloroquine. Supercoiled and relaxed pBluescript KS II⁺ (pKS II) are shown in lanes 7 and 8, respectively. The mobilities of the nicked, relaxed, and supercoiled forms of pBluescript KS II⁺ DNA are indicated on the right.

control incubation of topo70^{2XCys} with the DNA under reducing conditions (Fig. 2B) or after incubation of the WT enzyme with the DNA under oxidizing conditions (Fig. 2A). The detection of topo70^{2XCys} in a salt stable complex with the plasmid DNA after oxidization with GSSG, but not after incubation in the presence of DTT, indicated that the double mutant enzyme had been trapped on the DNA through an intramolecular disulfide crosslink between the cysteines at positions 367 and 499.

Analysis of Sucrose Gradient Fractions for the Presence of DNA-Associated and Free Topoisomerase I Activity. To confirm the presence of the topo70^{2XCys} on the DNA in the GSSG-treated sample and to investigate whether it retained enzymatic activity, a portion of fraction 8 from the sucrose gradient was assayed for relaxation activity on an exogenous substrate in the presence of DTT to release any disulfide crosslinked enzyme from the pRc/CMV DNA. The exogenous substrate used for this assay was supercoiled pBluescript KS II⁺ plasmid DNA (3.0 kb), which, by virtue of its small size relative to pRc/CMV DNA (5.5 kb), could easily be separated by agarose gel electrophoresis from the pRc/CMV DNA (not shown in the figures). As can be seen in Fig. 3A, lane 6, under reducing conditions, all of the pBluescript KS II⁺ DNA was completely relaxed. No fully relaxed pBluescript KS II⁺ DNA was observed when the gradient fraction was incubated in the absence of DTT (Fig. 3A, lane 5), suggesting that the sucrose gradient fraction contained no free enzyme. Likewise, in the control sample where topo70^{2XCys} had been maintained in the reduced state with DTT before sedimentation in sucrose, no enzyme activity was found to cosediment with the DNA even when the assay was carried out in the presence of DTT (Fig. 3A, lanes 3 and 4). Despite the absence of detectable protein by the immunoblot assay, a small amount of relaxation activity was observed cosedimenting with the pRc/CMV DNA in the control gradient for the WT enzyme that had been incubated with the DNA in the presence of GSSG. However, unlike the case of topo70^{2XCys}, this activity was detected both in the absence and presence of DTT (Fig. 3A, lanes 1 and 2). Although the origin of this WT activity is unknown, its

Fig. 4. Assay for covalent complex formation by topo70^{2XCys} on the endogenous pRc/CMV DNA. Aliquots of fraction 8 from the sucrose gradient purification of topo70^{2XCys}-pRc/CMV complexes were incubated under standard reaction conditions at 37°C in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of 10 µM camptothecin (CPT) without (lanes 2 and 3) or with (lanes 4 and 5) DTT. The reactions were stopped with SDS to trap enzyme-DNA covalent complexes and treated with proteinase K before agarose gel electrophoresis. Another aliquot of fraction 8 that had been stored at 4°C was treated with proteinase K and analyzed in lane 1 to establish the baseline level of nicked DNA in the sample. The mobilities of the nicked and topoisomer forms of pRc/CMV DNA are indicated on the left side.

detection clearly did not require reducing conditions as was the case for crosslinked topo70^{2XCys}. It is possible that it resulted from aggregated forms of the enzyme that sedimented near the pRc/CMV DNA in the gradient.

Because the experiments described below critically depend on the absence of free enzyme in the gradient fractions containing topo70^{2XCys} disulfide crosslinked to pRc/CMV, the presence of a trace amount of activity in the gradients for the WT enzyme prompted us to reassay the samples shown in Fig. 3A and carry out the agarose gel electrophoresis in the presence of chloroquine. Under these electrophoresis conditions, the topoisomers of the supercoiled substrate DNA are resolved, permitting the detection of even a very small shift toward the relaxed state. As can be seen in Fig. 3B (compare lanes 5 and 7), there was no detectable relaxation of the DNA after 30 min of incubation.

Assays for Cleavage Activity in Topo70^{2XCys}-pRc/CMV DNA Complexes. When topoisomerase I reactions are stopped with SDS, religation is blocked and a small fraction of the enzyme molecules is trapped in a covalent complex on the DNA (17). In the presence of the topoisomerase I poison, camptothecin, religation is impeded, which causes an increase in the amount of covalent complex produced by the SDS stop procedure (18, 19). The SDS cleavage assay was used to measure the DNA cleavage capability of topo70^{2XCys} enzyme that cosedimented with the pRc/CMV DNA. This assay differs from the plasmid relaxation assay because DNA cleavage can be detected without the requirement for DNA rotation that is inherent in the relaxation assay. A portion of sucrose gradient fraction 8 containing the purified topo70^{2XCys}-pRc/CMV complexes was incubated under standard topoisomerase I reaction conditions at 37°C in the absence or presence of camptothecin, and the reactions were stopped with SDS. The samples were treated with proteinase K to remove the bulk of the covalently bound protein before agarose gel electrophoresis so that any trapped covalent complexes migrated at the position of the nicked plasmid DNA in the gel. To establish the background level of preexisting nicked DNA in the sample, a control sample was prepared by using a portion of fraction 8 that had been stored at 4°C and was treated with proteinase K to inactivate the enzyme before the addition of the SDS (Fig. 4,

lane 1). In both the absence and presence of camptothecin, the amount of nicked DNA detected increased when compared with the control and, consistent with the mode of action of camptothecin, more covalent complex was observed in the presence of the drug (Fig. 4, compare lanes 2 and 3 with lane 1). These results indicate that the disulfide crosslinked enzyme was active on the endogenous pRc/CMV DNA in the complex, at least with respect to DNA cleavage. When topo70^{2XCys} was dissociated from the DNA with DTT before incubation at 37°C, less covalent complex was observed when the reactions were stopped with SDS (Fig. 4, lanes 4 and 5), presumably because the fraction of the total enzyme bound to the DNA at the time of addition of SDS in these samples was less than in those where the enzyme was disulfide crosslinked on the DNA.

DNA Rotation Occurs in the Closed Clamp Conformation of Topoisomerase I. The isolated topo70^{2XCys}-DNA complexes were used to test whether DNA rotation can occur in the context of the closed clamp form of the topoisomerase. Such a test requires an assay that depends on rotation of the DNA during the lifetime of the nicked intermediate. Thus, temperature shifts were used to alter the winding angle of the DNA helix (20, 21), and we asked whether the bound topo70^{2XCys} was capable of relaxing the resultant supercoils in the pRc/CMV DNA.

The original crosslinking of topo70^{2XCys} by GSSG in the presence of pRc/CMV DNA was carried out at 4°C, and, after purification by sucrose gradient centrifugation, the gradient fractions were maintained at 4°C. Thus, subsequent treatment of a portion of fraction 8 from the sucrose gradient with proteinase K at 4°C and analysis by agarose gel electrophoresis displayed a series of pRc/CMV topoisomers with gel mobilities reflecting the winding angle of the DNA at 4°C (Fig. 5A, lane 1). Incubation of the DNA sample at 23°C for 60 min in the absence of DTT, followed by a similar analysis revealed that a subset of the topoisomers had shifted to a slower mobility in the gel (Fig. 5A, lane 2). Moreover, this redistribution of topoisomers exactly corresponded to that observed for the entire population of topoisomers when the bound enzyme was released by treatment with DTT (Fig. 5A, compare lanes 2 and 3). A similar result was observed when the sample was incubated at 37°C, except that in this case, there was a greater reduction in the mobilities of the topoisomers as expected from the larger temperature shift (Fig. 5A, lanes 4 and 5). These results indicated that the enzyme molecules bound to the pRc/CMV DNA by virtue of the disulfide crosslink were capable of relaxing the supercoils that occurred as a result of the temperature shift, and therefore DNA rotation can occur within the confines of the closed clamp conformation of the enzyme.

We reproducibly observed that 30–50% of the topoisomer population exhibited an altered mobility in these temperature shift experiments, and we interpret this result as a reflection of the proportion of the pRc/CMV molecules containing bound and active disulfide crosslinked enzyme. The presence of a subset of topoisomers with unchanged mobilities even after a 60-min incubation at the higher temperatures (Fig. 5A, lanes 2 and 4) provides an internal control verifying that the observed relaxation for the remainder of the molecules does not result from contamination of the sample by free enzyme.

Although the results described above clearly showed that DNA rotation can occur without clamp opening, they did not address whether the rate of relaxation was comparable to that for the enzyme not constrained in the closed clamp configuration by the disulfide bond. Thus, we compared the time course for the relaxation of pRc/CMV DNA to which the enzyme was crosslinked with the rate after release of the enzyme by DTT. As can be seen in Fig. 5B (odd numbered lanes), even at the earliest time analyzed (15 s), the mobility shift for the subset of topoisomers containing bound enzyme was complete (45% shifted at

Fig. 5. Relaxation of pRc/CMV DNA by the cosedimenting topo70^{2XCys} enzyme. (A) An aliquot of fraction 8 from the sucrose gradient purification of topo70^{2XCys}-pRcCMV DNA complexes that had been stored at 4°C was added directly to proteinase K to establish the initial topoisomer distribution for the DNA (lane 1). Additional aliquots were incubated in the absence (lanes 2 and 4) or the presence (lanes 3 and 5) of DTT in reaction buffer at both 23°C and 37°C for 60 min as indicated in the figure, and the reactions were stopped with proteinase K. The agarose gel analysis was carried out in the presence of 0.3 μg/ml chloroquine to enhance the resolution of the topoisomers and separate the topoisomers from the nicked DNA. The mobilities of the various forms of pRc/CMV DNA are labeled as for Fig. 4. (B) A series of reactions containing a sample of fraction 8 from a sucrose gradient similar to one used for the analyses shown in A were initially incubated in the absence (odd numbered lanes) or presence (even numbered lanes) of DTT at 4°C for 60 min. For the zero time control, one of the reactions from each set was stopped by the addition of proteinase K (lanes 1 and 2). The slightly reduced mobility for the topoisomer population in lane 2 compared with lane 1 reflects the fact that the incubation conditions here are slightly different from those before purification through sucrose and, after release by DTT, the enzyme can act on the entire population of topoisomers. The remaining reactions were transferred to 37°C and the incubation was continued for the indicated lengths of time before being stopped by the addition of proteinase K (lanes 3–12). The agarose gel analysis was the same as for A.

15 s vs. 47% at 5 min). However, after release of the enzyme from the DNA by DTT, the mobility shift of the entire population was not completed until ≈2 min of incubation (Fig. 5B, even numbered lanes). The higher rate observed for the crosslinked enzyme in the absence of DTT is consistent with the view that the enzyme was irreversibly bound to the DNA and therefore acting in a completely processive manner. After release by DTT, relaxation occurred by a distributive mode, thus accounting for the slower observed rate. Because the bound and free enzyme molecules relaxed the DNA by two different mechanisms, a direct comparison of the relaxation rates is not feasible. However, in light of these results, it is highly unlikely that the rate of DNA rotation during relaxation by the disulfide clamped form of topo70^{2XCys} is severely impeded when compared with the rotation rate for the reduced form of the mutant enzyme, and it is possible that the rates are similar.

A mobility shift for the topoisomers containing bound topo70^{2XCys} was also observed after the incubation of the gradient fraction at 37°C in the experiment shown in Fig. 4. Notably, SDS cleavage in the presence of camptothecin resulted in a near complete depletion of the same subset of topoisomers that were shifted in mobility during the 37°C incubation (Fig. 4, compare lanes 2 and 3). This is the expected result because SDS-induced cleavage in the absence of DTT should be restricted to the subpopulation of topoisomers containing the

crosslinked enzyme and which therefore displayed an altered mobility after incubation at the higher temperature.

Conclusions

Although the crystal structure of human topoisomerase I seems compatible with a rotational model for the relief of supercoils during DNA relaxation, modeling studies indicated that the DNA would likely contact both the cap and the linker regions of the protein during the rotation process (5). Thus, it would seem that the protein must undergo a conformational change after cleavage to accommodate DNA rotation. Two proposals have been put forth for the nature of this conformational change. First, it was suggested that the space downstream of the cleavage site in the DNA could be expanded by the upward and downward displacement of the cap and the linker regions, respectively (5). Such opposing movements coupled with a slight tilt of the DNA could explain how rotation occurs. Alternatively, it has been suggested that, after cleavage, the entire cap region of the protein lifts upwards, analogous to what happens when the DNA dissociates from the enzyme, to create an opening large enough to accommodate the rotating DNA (Fig. 1D) (3). Our finding

that DNA relaxation efficiently occurs when the “lips” of the enzyme are held closed by a disulfide crosslink rules against the latter hypothesis and remarkably demonstrates that rotation is possible in the closed clamp conformation. Moreover, the kinetic analyses strongly suggest that the rate of rotation is not severely impeded when the enzyme is locked in the closed clamp conformation. Therefore, it would seem that only minor shifts in the positions of the cap and linker regions that surround the DNA downstream of the scissile phosphate are sufficient to accommodate DNA rotation. This apparent requirement for only minimal conformational adjustments during DNA relaxation by this type IB topoisomerase is striking in view of the relatively large conformational changes that accompany DNA topological manipulations by type IA and type II topoisomerases (3).

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