Topoisomerase II-mediated DNA Cleavage and Religation in the Absence of Base Pairing

ABASIC LESIONS AS A TOOL TO DISSECT ENZYME MECHANISM

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The interaction of topoisomerase II with its DNA cleavage site is critical to the physiological functions of the enzyme. Despite this importance, the specific enzyme-DNA interactions that drive topoisomerase II-mediated DNA cleavage and religation are poorly understood. Therefore, to dissect interactions between the enzyme and its cleavage site, abasic DNA lesions were incorporated into a bilaterally symmetrical and identical cleavage site. Results indicate that topoisomerase II has unique interactions with each position of the 4-base overhang generated by enzyme-mediated DNA cleavage. Lesions located 2 bases 3' to the point of scission stimulated cleavage the most, whereas those 3 bases from the point of scission stimulated cleavage the least. Moreover, an additive and in some cases synergistic cleavage enhancement was observed in oligonucleotides that contained multiple DNA lesions, with levels reaching >60-fold higher than the wild-type substrate. Finally, topoisomerase II efficiently cleaved and religated a DNA substrate in which apyrimidinic sites were simultaneously incorporated at every position on one strand of the 4-base overhang. Therefore, unlike classical DNA ligases in which base pairing is the driving force behind closure of the DNA break, it appears that for topoisomerase II, the enzyme is responsible for the spatial orientation of the DNA termini for ligation.

Eukaryotic topoisomerase II is an enzyme that is required for a number of indispensable nuclear processes, including DNA replication, recombination, and chromosome segregation (1–4). Moreover, it is the primary target for some of the most effective and commonly employed anticancer chemotherapy regimens used for the treatment of human malignancies (5–9). However, in order for topoisomerase II to fulfill its pivotal role in any of these processes, it must create double-stranded breaks in the genetic material.

Topoisomerase II generates these breaks by the concerted actions of two active site tyrosyl residues, each of which induces a transient, single-stranded nick in opposite strands of the DNA helix, such that a 4-base 5'-overhang is generated (10, 11). The integrity of the genetic material is preserved during the cleavage reaction by the formation of covalent phosphotyrosyl bonds between these residues and the newly generated 5' termini (12). Following ATP binding, a second helix is translocated through the topoisomerase II-associated double-stranded DNA break, and the cleaved DNA is religated (13–15).

Despite the importance of the DNA cleavage/religation reaction to the functions of topoisomerase II, relatively little is known regarding the specific enzyme-DNA interactions that drive this reaction. Although consensus DNA cleavage sequences have been reported for the eukaryotic enzyme, they are relatively weak and show little similarity to one another (8, 16). Thus, the molecular interactions that underlie the site specificity of topoisomerase II remain an enigma.

Previous attempts to dissect interactions between the enzyme and its cleavage site characterized the effects of DNA lesions (apurinic sites, apyrimidinic sites, or base mismatches) incorporated at specific positions in a topoisomerase II cleavage site (17–20). All of these lesions displayed a strict positional specificity. When they were located within the 4-base overhang generated by cleavage, levels of DNA scission increased 3–18-fold. Conversely, lesions located immediately outside the 4-base overhang inhibited DNA cleavage. Although position-specific variations in stimulatory DNA lesions were observed, conclusions regarding nucleotide positions within the 4-base overhang were limited because the relative effects of position (e.g. removal of a base at the point of scission versus one base away) as opposed to sequence (e.g. removal of a guanine versus an adenine) could not be distinguished.

To bypass these limitations and more fully define interactions between the enzyme and specific positions within its DNA cleavage site, abasic sites were incorporated into a topoisomerase II cleavage site in which the central bases were bilaterally symmetrical and identical (see Fig. 1). Because abasic lesions contained within a given strand of this cleavage site differed only by location rather than the type of base removed, this site allowed a systematic analysis of the 4-base overhang in topoisomerase II-mediated DNA cleavage/religation. Results indicate that topoisomerase II displays a positional preference, such that the efficacy of DNA lesions is dependent on their location within the cleavage site. In addition, the enzyme efficiently cleaves and religates a DNA substrate in which apyrimidinic sites are simultaneously incorporated at every position on one strand of the 4-base overhang, demonstrating that base pairing within the cleavage site is not required either for formation or closure of the DNA break.

**EXPERIMENTAL PROCEDURES**

*Preparation of Oligonucleotides—A 40-base single-stranded oligonucleotide that corresponds to residues 1072–1111 of the MLL oncogene (21, 22) and its complementary oligonucleotide were prepared by Cruachem Inc. The sequences of the top and bottom oligonucleotides were 5'-GCCTGGGTAGACAAGC| AAAAACTGCTCCTCGAAAAAATT-3' and 5'-AAATTTTTTTGGAGACAGTG | TTTGCTTTTTGCCAGG-3', respectively. The points of topoisomerase II-mediated DNA cleavage are denoted by the arrows. Single-stranded oligonucleotides containing a tetrahydrofuran abasic site analogue were prepared in a similar manner utilizing a tetrahydrofuran phosphoramidite. Single-
Topoisomerase II-medi­ated DNA Cleavage—Topoisomerase II-mediated DNA cleavage reac­tions were carried out by a pro­to­col simi­lar to that of Kin­gain et al. (19). Reac­tions con­tained 100 nm oligonucleotide in 19 μl of cleav­age buffer (10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 100 mM KCl, and 2.5% glycerol) that con­tained 5 mM MgCl2 and were ini­tiated by the addi­tion of 1 μl of human topoisomerase II (final con­cen­tra­tion, 150 μg). (Human topoisomerase II was pu­ri­fied from Saccharomyces cerevisiae as described previ­ously (19).) Reac­tions were incu­ba­tions for 10 min at 37 °C and stopped with 2 μl of 10% SDS fol­lowed by 1.5 μl of 250 mM EDTA. When ap­propriate, cleav­age reac­tions were reversed by the addi­tion of 1.5 μl of 250 mM EDTA for 5 min at 37 °C prior to the deter­gent. Cleav­age prod­ucts were diges­ted with pro­teinase K, pre­cip­i­ta­tions twice with etha­nol, and resol­ved by electropho­resis in denau­t­ur­ing 7 M urea, 14% poly­acry­lamide gels as described previ­ously (18). Reac­tion pro­ducts were visu­alized and quan­tified using a Phos­pho­Imager sys­tem. In all cases (other than those that incor­po­rated an abasic site on both strands), cleav­age was mon­i­tored on the com­ple­men­tary wild-type strand. Leve­l of DNA cleav­age were cal­cu­lated either rea­tive to that ob­tained with the wild-type sub­strate or from the per­cen­tage of total sub­strate that was cleaved.

Topoisomerase II-medi­ated DNA Religation—DNA religation as­says were car­ried by a mod­i­fication of the pro­ce­dure of Osheroff and Ze­ch­iedrich (22). Cleav­age/re­ligation equi­libria were estab­lished as described above in cleav­age buffer that con­tained 5 mM CaCl2. Kine­ti­cally com­petent topo­isomerase II-DNA cleav­age com­plexes were trapped by the addi­tion of EDTA (final con­cen­tra­tion, 6 mM). NaCl was added (final con­cen­tra­tion, 500 mM) to pre­vent re­cleav­age. Re­ligation was ini­tiated by the addi­tion of MgCl2 (final con­cen­tra­tion, 0.1 mM) and termi­nated by the addi­tion of 2 μl of 10% SDS at vari­ous times up to 60 s. Sam­ples were pre­pared and anal­yzed as des­cribed above. The appar­ent first or­der rate of DNA re­ligation was deter­mined by quan­tify­ing the loss of the cleav­ing prod­uct.

RESULTS

To dis­sect the rec­og­ni­tion of the DNA cleav­ing site by topo­isomerase II and the role of base pair­ing in the cleav­ing/reli­gation reac­tion of the enzy­me, abasic les­sions were incor­po­rated into a 40-base pair oligonucleotide that corre­sponds to resi­dues 1072–1111 of the MLL gene and includes a topo­isomerase II cleav­ing site at nucleo­tide pos­i­tion 1087 (21, 22). This bi­lar­ty sym­met­ri­cal­ly site was used be­cause it con­tains iden­ti­cal bases along each strand of the 4-base over­hang (i.e., all ade­nine res­i­dus on the top strand and thy­mine res­i­dus on the bot­tom) (Fig. 1). The posi­tions of DNA les­sions are de­signed rela­tive to the point of cleav­ing with the points of scis­sion on both the top and bot­tom strands locat­ed 5’ to the +1 base. Cleav­ing of the top and bot­tom stran­ds of this 5’-la­beled oligonucleotide by hu­man topo­isomerase II res­ults in the for­ma­tion of ra­dioac­tive 16- and 20-base prod­ucts, re­spec­tive­ly, such that a 4-base 5’-cleav­ing over­hang is gen­er­ated.

Topoisomerase II Dis­plays a Posi­tional Pre­ference for DNA Les­sions in the 4-base Over­hang—The sen­si­tiv­ity of hu­man topo­isomerase II toward alter­a­tions at specif­ic posi­tions in the 4-base cleav­ing over­hang was char­ac­terized by incor­po­rat­ing abasic DNA les­sions at each posi­tion within the top and bot­tom stran­ds (Fig. 1). Con­sis­tent with our pre­vious work (17, 19), each in­di­vid­ual les­sion stimu­lated enzy­me-medi­ated DNA cleav­ing, and great­er levels of cleav­ing were ob­served with apurinic sites (top strand) than with apyrimidinic sites (bot­tom strand). How­ever, levs of cleav­ing stimu­la­tion for ei­ther type of lesion were not uni­form across the cleav­ing site. Consis­tent­ly, lesions at the +2 posi­tion stimu­lated DNA scis­sion the most (~9–11-fold above the wild-type sub­strate. In con­trast to the strong pre­ference for the +2 posi­tion, only a weak pre­ference (~2–3-fold above wild-type) for the +3 posi­tion was de­tected on both stran­ds. The preference for les­sions placed at the +1 ver­sus +4 posi­tions was irreg­u­lar. When com­par­ing these two posi­tions, a slight pre­ference for the +1 posi­tion was ob­served on the top strand, whereas on the bot­tom strand the +4 posi­tion was preferred. As dis­cussed below, these find­ings sug­gest a pre­dispo­si­tion of the enzy­me for the “left side” of the cleav­ing site.

Although the cleav­ing site uti­lized in this study per­mits a unique ana­lysis of the posi­tional pre­ferences of the enzy­me, the gen­er­al­ity of these con­clu­sions must be tem­pered by the fact that only a sin­gle site was ex­am­ined. How­ever, these pre­fer­ences are sup­ported by re­sults ob­tained with at least two other DNA cleav­ing sites (19).1 Even with the above cave­at, it is clear that topo­isomerase II does not have uni­form in­ter­ac­tions with each base of the over­hang; rather the enzy­me ap­pears to have a posi­tional pre­ference for DNA les­sions and dis­plays spe­ci­fic re­sponses to alter­ations at each posi­tion.

Multiple Abasic DNA Les­sions Stu­mat­es Topoisomerase II-Medi­ated DNA Cleav­ing—Sev­er­al clin­i­cal­ly rele­vant anti­can­cer drugs exer­cize their che­mo­thera­peu­tic ac­tions by in­creas­ing lev­els of co­valent topo­isomerase II-cleaved DNA com­plexes (6–9). As a re­sult of their ac­tions, drugs that “poison” topo­isomerase II (i.e., shift the cleav­ing/re­ligation equi­lib­rium to­ward the cleav­ing state) in­crease lev­els of pro­tein-as­so­ci­ated DNA breaks and are le­thal to ra­di­ally di­vid­ing can­cer cells. The or­i­nal mod­els pro­posed for the ac­tions of these poisons gen­er­al­ly placed a drug mole­cule at the point of scis­sion on each strand of the dou­ble helix with two mole­cules ac­tion­ing in con­cert to stimu­late dou­ble-stran­ded DNA cleav­ing (6, 8, 16, 24–26).

Based on the re­sults of pre­vious stud­ies with DNA les­sions, an alter­na­tive mod­el known as the “pos­i­tional poison mod­el” was pro­posed that encompassed the ac­tions of both anti­can­cer drugs and DNA les­sions (18). Un­like pre­vious drug mod­els, the posi­tion­al poison mod­el sug­gested that two drug mole­cules were not re­quired to stimu­late dou­ble-stran­ded DNA cleav­ing, be­cause a sin­gle, strand-spe­cific DNA les­sion was capa­ble of en­hanc­ing enzy­me-medi­ated DNA cleav­ing at both points of scis­sion. There­fore, to ex­tend our use of DNA les­sions as a probe of drug me­cha­nism, a se­ries of oligonucleo­tides con­taining two abasic sites was ana­lyzed to deter­mine whether mul­ti­ple les­sions might also dis­play a posi­tion­al pre­ference.

In most cases, the stimu­la­tory ef­fects of mul­ti­ple DNA les­sions ap­peared to be at least additive (i.e., lev­els of cleav­ing 1 S. D. Cline and N. Osheroff, unpub­lished re­sults.)
DNA lesions on both the top (monitored on the complementary wild-type strand. For substrates with other (17), separated by a gap, or located at both points of observed when abasic sites were positioned adjacent to each the theoretical sum of DNA cleavage levels for the individual lesions are shown (open bars). Data represent the averages of three independent experiments. Standard deviations are indicated by error bars.

with two DNA lesions were as high as the theoretical sum of cleavage levels observed with the individual abasic sites) (Fig. 2, compare closed and open bars). This additive effect was observed when abasic sites were positioned adjacent to each other (17), separated by a gap, or located at both points of scission in either the +1 top/+1 bottom or the +4 top/+4 bottom positions (the postulated locations of drugs (6, 8, 16, 24–26)). Therefore, although only one topoisomerase II poison is required to induce double-stranded DNA cleavage, two appear to produce greater levels of cleavage enhancement.

Remarkably, in some cases a synergetic effect was observed. When lesions were incorporated at the +1/+2 or the +2/+3 positions on the top strand or the +2/+3 or the +3/+4 positions on the bottom strand levels of cleavage stimulation reached >60-fold and were ~2–3-fold greater than the predicted additive effects of the individual DNA lesions. Because this superadditive effect was only observed with abasic lesions at these positions, it appears that synergism within this cleavage site is dependent on two primary factors. First, the abasic sites must be located adjacent to each other. Second, lesions must be positioned on the left side of the cleavage site, such that the +1 or +2 positions on the top strand or the +3 or +4 positions on the bottom strand are included.

Topoisomerase II-mediated DNA Cleavage and Religation in the Absence of Base Pairing—Previous work by Westergaard and co-workers examined the importance of base pairing in ligation reactions between a topoisomerase II-cleaved DNA complex and external, acceptor fragments of DNA (27). In this intermolecular ligation reaction, base pairing of the external fragment to the cleaved DNA was important for the positioning of the incoming termini and enhanced rates of ligation as much as 8-fold. In contrast, studies that incorporated single abasic sites or base mismatches within a topoisomerase II cleavage site indicated that intramolecular DNA religation mediated by the enzyme (i.e. resealing the original DNA break covalently linked to topoisomerase II) was not inhibited by disruption of one of the 4 base pairs within the overhang (17, 18). Therefore, to further define the importance of base pairing within the 4-base overhang, enzyme-mediated DNA cleavage and intramolecular religation were examined in the presence of multiple abasic sites.

As seen in Fig. 3, topoisomerase II tolerated the removal of all four pyrimidines on the bottom strand (apyrimidinic sites were used because they are less disruptive to the structure of the double helix than apurinic sites (28–30)). In fact, cleavage increased significantly with the removal of each successive base and reached levels >60-fold above the wild-type substrate when all 4 bases on the bottom strand were removed.

Despite these dramatic increases in DNA scission, ~90% of the cleavage was reversed by the addition of EDTA, even when all the base pairing was removed from the 4-base overhang (Fig. 3). In addition, under conditions in which the enzyme was capable of carrying out DNA strand passage (i.e. in the presence of ATP), cleavage was still reversible in the absence of base pairing. Therefore, base pairing within the overhang is not required for either DNA cleavage or religation mediated by topoisomerase II.

Although not necessary for religation, the potential contributions of base pairing to the rate of this reaction are unknown. Therefore, the kinetics of religation were examined using a series of apyrimidinic substrates (Fig. 4). Removal of one (+1 position) or two bases (either at the +1/+2 or +3/+4 positions) from the bottom strand did not inhibit rates of religation. In fact, rates appeared to increase slightly, which is consistent with earlier findings with single DNA lesions (17). Removal of 3 or 4 bases decreased rates of religation ~2–3-fold, a relatively minor effect when compared with the >60-fold stimulation of cleavage induced by four apyrimidinic sites. It is unclear whether this small decrease in the rate of religation reflects a minor contribution of base pairing to this reaction or whether it reflects a lesion-induced structural distortion of the cleavage site in addition to the potential effects of base pairing. This notwithstanding, it is clear that under the conditions used in this system, base pairing is not the driving force behind topoisomerase II-mediated DNA religation.

**DISCUSSION**

Determining the specific molecular interactions that govern the topoisomerase II-mediated DNA cleavage/religation reac-
Abasic Sites as a Tool to Dissect Topoisomerase II Mechanism

Results indicate that topoisomerase II has a positional preference for DNA lesions located within the 4-base cleavage overhang. Although every DNA lesion introduced within this region stimulated DNA scission, levels of cleavage with abasic overhangs were set to 100%. The apparent first order rates of religation were determined by monitoring the loss of the cleavage product. Data represent the averages of two independent experiments.

In summary, the interaction of topoisomerase II with its DNA cleavage site is critical to the physiological and therapeutic functions of the enzyme. DNA lesions are rapidly becoming a powerful tool for understanding the factors that dictate these interactions.

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