Ponta, H., Rahmsdorf, H. J., Pai, S. H., Hirsch-Kauffmann, M., Herrlich, P., & Schweiger, M. (1974) *Mol. Gen. Genet. 134*, 281–297.
Shimamoto, N., & Wu, C.-W. (1980) *Biochemistry 19*, 842–848.
Shimamoto, N., Wu, F., & Wu, C.-W. (1981) *Biochemistry 20*, 4745–4755.
Shimamoto, N., Kamigochi, T., & Utiyama, H. (1986) *J. Biol. Chem. 261*, 11859–11865.
Stuber, D., & Bujard, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 167–171.
Zillig, W., Fujiki, H., Blum, W., Janekovic, D., Schweiger, M., Rahmsdorf, H. J., Ponta, H., & Hirsch-Kauffmann, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2506–2510.

Effects of Antineoplastic Drugs on the Post-Strand-Passage DNA Cleavage/Religation Equilibrium of Topoisomerase II†

Megan J. Robinson and Neil Osheroff*
Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146
Received August 23, 1990; Revised Manuscript Received November 15, 1990

ABSTRACT: The post-strand-passage DNA cleavage/religation equilibrium of *Drosophila melanogaster* topoisomerase II was examined. This was accomplished by including adenyl-5′-yl imidodiphosphate, a nonhydrolyzable ATP analogue which supports strand passage but not enzyme turnover, in assays. Levels of post-strand-passage enzyme-mediated DNA breakage were 3–5 times higher than those generated by topoisomerase II prior to the strand-passage event. This finding correlated with a decrease in the apparent first-order rate of topoisomerase II mediated DNA religation in the post-strand-passage cleavage complex. Since previous studies demonstrated that antineoplastic drugs stabilize the pre-strand-passage cleavage complex of topoisomerase II by impairing the enzyme's ability to religate cleaved DNA [Osheroff, N. (1989) *Biochemistry 28*, 6157–6160; Robinson, M. J., & Osheroff, N. (1990) *Biochemistry 29*, 2511–2515], the effects of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and etoposide on the enzyme's post-strand-passage DNA cleavage complex were characterized. Both drugs stimulated the ability of topoisomerase II to break double-stranded DNA after strand passage. As determined by two independent assay systems, *m*-AMSA and etoposide stabilized the enzyme's post-strand-passage DNA cleavage complex primarily by inhibiting DNA religation. These results strongly suggest that both the pre- and post-strand-passage DNA cleavage complexes of topoisomerase II serve as physiological targets for these structurally disparate antineoplastic drugs.

Physiological processes such as DNA replication, transcription, and recombination generate a number of topological structures in nucleic acids which must be resolved in order for the cell to transmit its genetic material faithfully from one generation to the next (Vosberg, 1985; Wang, 1985). In the eukaryotic cell, topological relationships in DNA are modulated by highly conserved enzymes known as topoisomerases (Vosberg, 1985; Wang, 1985; Osheroff, 1989a). The type II topoisomerase is an essential enzyme (DiNardo et al., 1984; Goto & Wang, 1984; Uemura & Yanagida, 1984; Holm et al., 1985) which is required for the segregation of daughter chromosomes (DiNardo et al., 1984; Uemura & Yanagida, 1984, 1986; Holm et al., 1985; Uemura et al., 1987) and the maintenance of proper chromosome structure (Berrios et al., 1985; Earnshaw & Heck, 1985; Earnshaw et al., 1985; Gasser & Laemmli, 1986; Gasser et al., 1986).

The catalytic cycle of topoisomerase II can be broken into a number of discrete steps (Osheroff, 1989a). (1) In the absence of either a divalent cation or ATP, the enzyme is capable of forming a noncovalent complex with its DNA substrate. (2) If a divalent cation (magnesium is used in vivo) is present, topoisomerase II rapidly establishes a double-stranded DNA cleavage/religation equilibrium prior to strand passage. When the DNA is cleaved, topoisomerase II is covalently attached to the newly generated 5′ termini. (3) Upon ATP binding, the enzyme passes an intact DNA helix through the transient break in the nucleic acid backbone. (4) Following this strand-passage event, topoisomerase II once again establishes a DNA cleavage/religation equilibrium. (5) Hydrolysis of the bound ATP cofactor triggers (6) enzyme turnover and allows topoisomerase II to initiate a new round of catalysis.

Recent studies indicate that topoisomerase II is the primary cellular target for a number of antineoplastic drugs (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989) which are widely used for the clinical treatment of human cancers (Cassileth & Gale, 1986; van Maanen et al., 1988; Fleming et al., 1989). The chemotherapeutic efficacies of the above agents correlate with their abilities to stabilize covalent topoisomerase II–DNA cleavage complexes (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). This drug-induced shift in the enzyme's DNA cleavage/religation equilibrium toward the cleavage event has been observed both in the absence (Chen et al., 1984; Pommier et al., 1985; Osheroff, 1989b; Robinson & Osheroff, 1990) and in the presence of ATP (Nelson et al., 1984; Tewey et al., 1984; Yang et al., 1985; Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). From studies performed in the absence of the enzyme's high-energy cofactor, it is clear that antineoplastic agents alter the DNA cleavage/religation equilibrium of topoisomerase II which occurs prior to strand passage. Recent

†This work was supported by National Institutes of Health Grant GM-33944 and North Atlantic Treaty Organization Grant 5-2-05/RG 0157188. M.J.R. was a trainee under Grant 5 T32 CA-09582 from the National Cancer Institute. For a portion of this work, N.O. was supported by Faculty Research Award FRA-370 from the American Cancer Society.

0006-2960/91/0430-1807$02.50/0

work indicates that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA)[1] (Robinson & Osheroff, 1990) and etoposide (Osheroff, 1989b), two potent topoisomerase II targeted drugs, effect this pre-strand-passage equilibrium primarily by inhibiting the enzyme's DNA religation event. However, since cells contain ATP and DNA strand passage is required for the catalytic function of topoisomerase II (Vosberg, 1985; Wang, 1985; Osheroff, 1989a), experiments carried out in the absence of a nucleotide triphosphate may not be completely representative of drug action in vivo. Unfortunately, studies performed in the presence of ATP are difficult to interpret from a mechanistic point of view. Because ATP can support topoisomerase II turnover in addition to DNA strand passage (Vosberg, 1985; Wang, 1985; Osheroff, 1989a), it is not clear whether the drug-induced increase in cleavage is due to the stabilization of the enzyme's pre- or post-strand-passage cleavage complex (or both).

Before the physiological mechanism of topoisomerase II targeted drugs can be completely described, their effects on each step of the enzyme's catalytic cycle must be well characterized. The present study describes the effects of antineoplastic agents on the post-strand-passage DNA cleavage/religation equilibrium of topoisomerase II. To accomplish this, the actions of drugs were assessed in the presence of APP(NH)P. This ATP analogue promotes topoisomerase II mediated DNA strand passage, but the nonhydrolyzable nature of its β,γ-imidodiphosphate bond will not permit the enzyme to undergo turnover and subsequent rounds of catalysis (Osheroff et al., 1983). Thus, in the presence of APP(NH)P, the post-strand-passage DNA cleavage/religation equilibrium predominates. Results of the present study indicate that both *m*-AMSA and etoposide stabilize the post-strand-passage topoisomerase II–DNA cleavage complex. Moreover, these drugs act primarily by inhibiting the enzyme's ability to religate cleaved DNA.

Experimental Procedures

DNA topoisomerase II was purified from the nuclei of *Drosophila melanogaster* Kc tissue culture cells or 6–12-h-old embryos by the procedure of Shelton et al. (1983). Negatively supercoiled bacterial plasmid pBR322 DNA (Bolivar et al., 1977) was obtained from *Escherichia coli* DH1 by a Triton X-100 lysis followed by double banding in cesium chloride–ethidium bromide gradients (Maniatis et al., 1982). *m*-AMSA was the generous gift of Dr. Yves Pommier, Laboratory of Molecular Pharmacology, NCI. The drug was stored at −20 °C as a 10 mM solution in DMSO (Pierce). Etoposide (VePesid, VP-16-23) was purchased from Bristol Laboratories as a sterile 20 mg/mL solution in etoposide diluent [2 mg/mL citric acid, 30 mg/mL benzyl alcohol, 80 mg/mL polysorbate 80/Tween 80, 650 mg/mL poly(ethylene glycol) 300, and 30.5% (v/v) ethanol]. The drug was stored at room temperature as per the manufacturer's instructions. Tris, ethidium bromide, and APP(NH)P were obtained from Sigma; analytical reagent grade $MgCl_2 \cdot 6H_2O$ was from Fisher, and SDS and proteinase K were from E. Merck Biochemicals. All other chemicals were analytical reagent grade.

Topoisomerase II Mediated DNA Cleavage. Unless stated otherwise, DNA cleavage reactions contained 25–100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total volume of 20 μL of cleavage buffer (10 mM Tris-HCl, pH 7.9, 25 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2.5% glycerol). Reactions which monitored the DNA cleavage/religation equilibrium established prior to the enzyme's strand-passage event contained no ATP analogue. Reactions which monitored the DNA cleavage/religation equilibrium established after enzyme-mediated strand passage contained 1 mM APP(NH)P. In all cases, samples were incubated at 30 °C for 6 min, and cleavage products were trapped (Gale & Osheroff, 1990) by the addition of 2 μL of 10% SDS. One microliter of 250 mM EDTA and 2 μL of a 0.8 mg/mL solution of proteinase K were added, and samples were incubated at 37 °C for 30 min to digest the topoisomerase II. Final products were mixed with 2.5 μL of loading buffer (60% sucrose, 0.05% bromophenol blue, 0.05% xylene cyanole FF, and 10 mM Tris-HCl, pH 7.9), heated at 70 °C for 1 min, and subjected to electrophoresis in 1% agarose (MCB) gels in 40 mM Tris–acetate, pH 8.3, and 2 mM EDTA. Following electrophoresis, DNA bands were stained in a 1 μg/mL solution of ethidium bromide and visualized by transillumination with ultraviolet light (300 nm). In some experiments, ethidium bromide (0.5 μg/mL) was added to both the gel and the running buffer prior to electrophoresis in order to facilitate the separation of DNA cleavage products from relaxed topoisomers. DNA bands were photographed through Kodak 23A and 12 filters using Polaroid type 665 positive/negative film. The amount of DNA was quantitated by scanning negatives with a Biomed Instruments Model SL-504-XL scanning densitometer. Under the conditions employed, the intensity of the bands in the negative was directly proportional to the amount of DNA present.

The effects of antineoplastic drugs on both the pre- and post-strand-passage DNA cleavage/religation equilibria of topoisomerase II were examined over a range of 0–120 μM *m*-AMSA or 0–100 μM etoposide. An amount of DMSO or etoposide diluent equal to that in drug-containing samples was added to all control samples. Neither diluent affected the topoisomerase II mediated DNA cleavage/religation equilibrium.

Topoisomerase II Mediated DNA Religation. Reactions contained 25–100 nM topoisomerase II and 5 nM negatively supercoiled pBR322 DNA in a total of 20 μL of cleavage buffer. Initial DNA cleavage/religation equilibria were established at 30 °C for 6 min. As described above, pre-strand-passage cleavage/religation equilibria were established in the absence of a nucleotide triphosphate, and post-strand-passage equilibria were established in the presence of 1 mM APP(NH)P. Topoisomerase II mediated religation of cleaved DNA was induced by either of the following two methods:

(1) Samples were rapidly shifted from 30 to 55 °C. Religation was terminated by the addition of SDS (1% final concentration) at various time points up to 30 s.

(2) Samples were rapidly shifted from 30 to 0 °C. Religation was terminated by the addition of SDS (1% final concentration) at various time points up to 120 s.

Following either procedure, samples were treated with EDTA and proteinase K as described above. Reaction products were resolved by agarose gel electrophoresis and quantitated as discussed in the previous section.

The effects of antineoplastic drugs on topoisomerase II mediated DNA religation were examined by adding either 60 μM *m*-AMSA or 100 μM etoposide to reactions prior to temperature shifts. Control reactions contained an amount of DMSO or etoposide diluent equivalent to that in the drug-containing samples.

[1] Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; etoposide, demethylepipodophyllotoxin thenylidene-β-D-glucoside; APP(NH)P, adenyl-5′-yl imidodiphosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

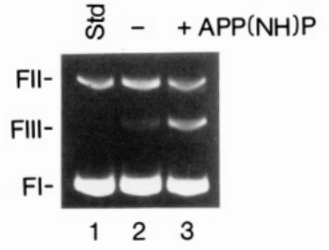

FIGURE 1: Effect of APP(NH)P on topoisomerase II mediated DNA cleavage/religation equilibria. Assays utilized 50 nM enzyme and were carried out as described under Experimental Procedures. An agarose gel is shown. Lane 1, DNA standard; lane 2, DNA cleavage carried out in the absence of APP(NH)P; lane 3, DNA cleavage carried out in the presence of 1 mM APP(NH)P. The positions of fully supercoiled DNA (form I, FI), nicked circular molecules (form II, FII), and linear molecules (form III, FIII) are indicated.

RESULTS

While ATP binding induces topoisomerase II to carry out its DNA strand-passage event, hydrolysis of the bound cofactor is required for enzyme turnover (Osheroff et al., 1983; Osheroff, 1986). Therefore, by employing APP(NH)P, a nonhydrolyzable ATP analogue, in reaction mixtures, it is possible to segregate the enzyme's post-strand-passage DNA cleavage/religation equilibrium from those catalytic steps which occur prior to strand passage.

The ability of APP(NH)P to induce topoisomerase II mediated strand passage is evidenced by the conversion of negatively supercoiled pBR322 plasmid DNA to a series of covalently closed relaxed topoisomers (see Figure 6) (Osheroff et al., 1983). In the presence of 50 nM *Drosophila* topoisomerase II and 1 mM APP(NH)P, the average linking difference (i.e., the number of superhelical twists) of 5 nM pBR322 DNA decreased by 15, from −25 to −10.[2] Since topoisomerase II removes two superhelical twists per strand-passage event (Vosberg, 1985; Wang, 1985; Osheroff, 1989a), the theoretical maximum change in the average linking difference of pBR322 produced by a 10-fold molar excess of enzyme would be 20. However, this theoretical maximum would be achieved only if (1) every molecule of topoisomerase II in the enzyme preparation was active, (2) every molecule of active enzyme in the reaction mixture catalyzed a productive strand-passage event, and (3) every strand-passage event was catalyzed in the same direction (i.e., toward a smaller linking difference). Considering that the decrease in the average linking difference obtained experimentally was 15 (75% of the theoretical maximum), it is clear that a large majority of topoisomerase II–DNA complexes formed in the presence of APP(NH)P must have undergone strand passage.

Characterization of the Post-Strand-Passage DNA Cleavage/Religation Equilibrium of Topoisomerase II. As compared to its pre-strand-passage DNA cleavage/religation equilibrium, *Drosophila* topoisomerase II mediates DNA breakage more efficiently following strand passage (Figure 1). This finding supports a similar observation reported by Osheroff (1986). It should be noted that the gel shown in Figure 1 was subjected to electrophoresis in the presence of ethidium bromide (0.5 μg/mL). The inclusion of this intercalating dye alters the electrophoretic mobility of all covalently closed DNA topoisomers such that they comigrate as a single band with the negatively supercoiled plasmid substrate (Maniatis et al., 1982). This procedure allowed the linear DNA molecules generated by topoisomerase II mediated cleavage to be separated from the partially relaxed DNA topoisomers produced by strand passage. [For a typical distribution of partially relaxed DNA topoisomers in assays which contained APP(NH)P, see Figure 6.]

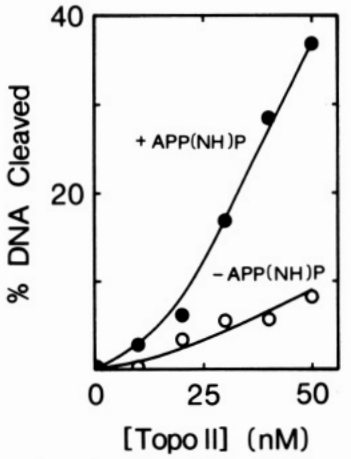

FIGURE 2: Effect of topoisomerase II concentration on the enzyme's pre-strand-passage and post-strand-passage DNA cleavage/religation equilibria. Assays were carried out as described under Experimental Procedures. Pre-strand-passage reactions (open circles) contained no APP(NH)P; post-strand-passage reactions contained 1 mM APP(NH)P. Data represent the average of three independent experiments.

To further characterize the levels of cleaved DNA generated by topoisomerase II before and after strand passage, cleavage/religation equilibria were examined over a wide range of enzyme concentrations (Figure 2). Over the concentration range employed, topoisomerase II cleaved 3–5 times more DNA after strand passage had occurred.

In order to determine whether the greater level of DNA breakage observed following strand passage was due primarily to an increase in the enzyme's rate of cleavage or a decrease in its rate of religation, the first-order kinetics of topoisomerase II mediated DNA religation were characterized. To accomplish this, a time dependent DNA religation assay was utilized. This assay takes advantage of the finding that the religation activity of *Drosophila* topoisomerase II is less sensitive to variations in temperature than is its cleavage activity. While the enzyme displays a decreased ability to cleave double-stranded DNA at extremes of temperature (i.e., 0 or 55 °C), it can still religate cleaved nucleic acids (M. J. Robinson and N. Osheroff, unpublished results). Thus, following a shift from 30 to 55 °C, linear DNA molecules generated by topoisomerase II mediated DNA cleavage are reconverted to their original supercoiled state in a time-dependent fashion. Heat-induced religation of DNA by topoisomerase II has been demonstrated previously for the *Drosophila* (Osheroff & Zechiedrich, 1987), human (Hsiang & Liu, 1989), and calf thymus (Hsiang et al., 1989) enzymes in vitro and the mouse enzyme (Hsiang & Liu, 1989) in vivo.[3]

Heat-induced DNA religation assays were performed as follows. Mixtures of topoisomerase II, negatively supercoiled pBR322 DNA, and $MgCl_2$ were incubated at 30 °C in the

[2] Reaction products were resolved by electrophoresis in a 1% agarose gel as described under Experimental Procedures. Fully supercoiled (FI) pBR322 DNA was resolved into its individual topoisomers by electrophoresis in a 1% agarose gel which contained 2.5, 3, or 4 μM chloroquine (Sander et al., 1987). In all cases, the average linking difference of DNA samples was determined by densitometric scanning.

[3] A topoisomerase II mediated DNA religation assay which utilizes EDTA to trap cleavage complexes formed in the presence of $CaCl_2$ also has been described (Osheroff & Zechiedrich, 1987; Zechiedrich et al., 1989). However, this assay could not be used to study the enzyme's post-strand-passage DNA religation activity, because Ca·ATP will not support the strand-passage event mediated by *Drosophila* topoisomerase II (Osheroff et al., 1983; Osheroff & Zechiedrich, 1987).

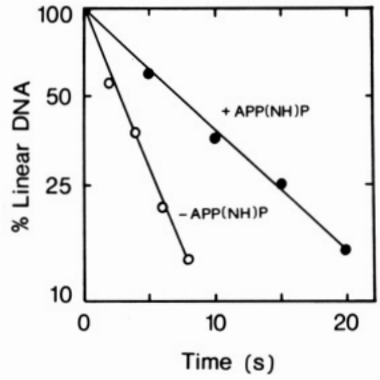

FIGURE 3: Effect of strand passage on the ability of topoisomerase II to religate cleaved DNA. Assays were carried out as described under Experimental Procedures. Religation was initiated by shifting reaction mixtures from 30 to 55 °C. A semilogarithmic plot of percent linear DNA versus time is shown. Results are plotted as the loss of linear DNA in the absence (pre-strand-passage, open circles) or presence (post-strand-passage, closed circles) of 1 mM APP(NH)P. The percent linear DNA was arbitrarily set to 100% at time zero. Data represent the average of three independent experiments.

absence or presence of APP(NH)P to establish pre- or post-strand-passage DNA cleavage/religation equilibria, respectively. At time zero, samples were shifted to 55 °C. Enzyme-mediated religation was stopped at various time points by the addition of SDS.

A previous study on the pre-strand-passage religation activity of topoisomerase II demonstrated that the enzyme rejoins double-stranded DNA breaks one strand at a time (Zechiedrich et al., 1989). In the first event, topoisomerase II converts linear DNA to nicked molecules. In the second event (which proceeds at one-sixth the rate of the first), topoisomerase II converts nicked molecules to covalently closed circular DNA. A similar reaction pathway was observed for post-strand-passage religation. By utilization of the assay described above, the pre- and post-strand-passage religation activities of topoisomerase II were compared (Figure 3). As determined by the loss of linear DNA (i.e., the enzyme's first religation event), the apparent first-order rate of post-strand-passage DNA religation was approximately 3-fold slower than that determined for the pre-strand-passage reaction. A similar result was obtained for the enzyme's second religation event (not shown). Therefore, the enhanced DNA breakage observed after strand passage correlates with a decreased ability of topoisomerase II to religate cleaved DNA.

Effect of Antineoplastic Drugs on the Post-Strand-Passage DNA Cleavage/Religation Equilibrium of Topoisomerase II. Antineoplastic drugs stabilize the topoisomerase II–DNA cleavage complex which is formed prior to strand passage (i.e., in the absence of a nucleoside triphosphate cofactor) approximately 5–10-fold (Chen et al., 1984; Pommier et al., 1985; Osheroff, 1989b; Robinson & Osheroff, 1990). Previous studies indicate that *m*-AMSA and etoposide stabilize this complex primarily by inhibiting the enzyme's ability to religate cleaved DNA molecules (Osheroff, 1989b; Robinson & Osheroff, 1990). Since as described above, the post-strand-passage topoisomerase II–DNA cleavage complex is 3–5 times more stable than its pre-strand-passage counterpart, it is not clear whether drugs will show the same potency toward complexes which are already stabilized.

To address this point, Figure 4 compares the relative effects of *m*-AMSA and etoposide on the DNA cleavage/religation equilibria of topoisomerase II which occur before [−APP(NH)P] and after [+APP(NH)P] strand passage. Although both drugs enhanced post-strand-passage DNA breakage, two

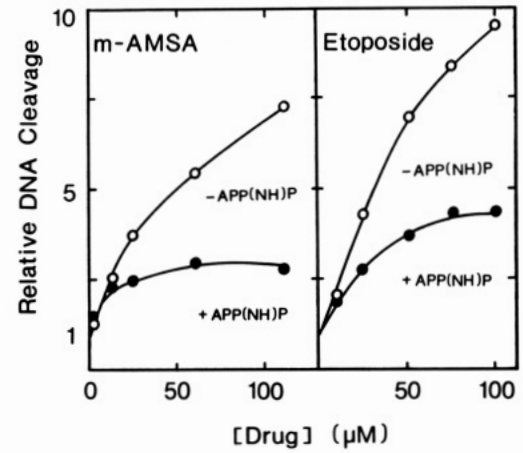

FIGURE 4: Effect of strand passage on the ability of antineoplastic drugs to induce topoisomerase II mediated DNA cleavage. Assays were carried out as described under Experimental Procedures. Results are plotted as the relative amount of double-stranded DNA cleavage versus the concentration of *m*-AMSA (left panel) or etoposide (right panel). The relative level of DNA cleavage was arbitrarily set to 1 in the absence of drug. Pre-strand-passage reaction (open circles) assays contained no APP(NH)P; post-strand-passage assays (closed circles) contained 1 mM APP(NH)P. Data represent the average of two to four independent experiments.

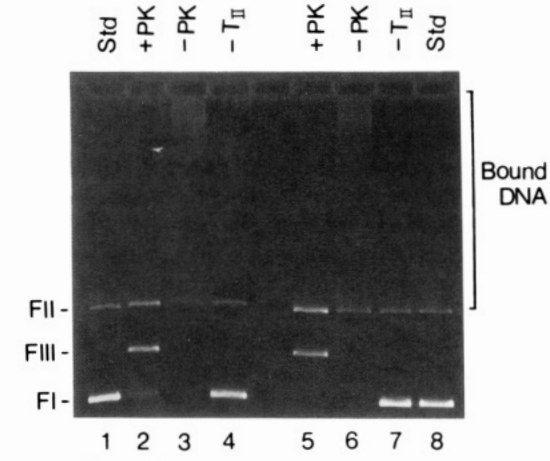

FIGURE 5: Effects of *m*-AMSA (lanes 1–4) and etoposide (lanes 5–8) on the post-strand-passage DNA cleavage/religation equilibrium are mediated by topoisomerase II. Assays were carried out in the presence of 60 μM *m*-AMSA or 100 μM etoposide as described under Experimental Procedures. Lanes 1 and 8, DNA standards; lanes 2 and 5, DNA cleavage products were digested with proteinase K prior to electrophoresis; lanes 3 and 6, DNA cleavage products were not digested with proteinase K; lanes 4 and 7, assays were carried out in the absence of topoisomerase II. The positions of topoisomerase II bound DNA as well as those of free form I, II, and III molecules are indicated.

differences were apparent. (1) Levels of post-strand-passage DNA breakage plateaued at considerably lower drug concentrations. (2) The relative abilities of drugs to stimulate DNA breakage decreased following strand passage.[4] It should be noted, however, that initial levels of post-strand-passage DNA breakage obtained in the absence of drugs were 3–5 times higher than levels obtained before strand passage (see Figure 2). Therefore, while the relative potency of drugs was decreased, the actual levels of drug-induced DNA breakage which followed strand passage were greater than those observed in pre-strand-passage reactions.

[4] The apparent decrease in drug potency which is observed following strand passage cannot be ascribed to a depletion of the initial supercoiled DNA substrate. Results similar to those shown in Figure 4 were obtained when maximally 25–30% of the supercoiled substrate was cleaved.

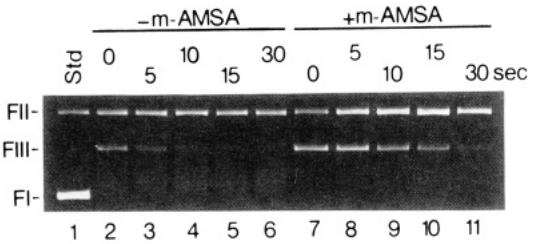

FIGURE 6: Effect of *m*-AMSA on the ability of topoisomerase II to mediate post-strand-passage DNA religation at 55 °C. Religation was initiated by shifting assay mixtures from 30 to 55 °C as described under Experimental Procedures. An agarose gel run in the absence of ethidium bromide is shown. Assays were carried out in the absence (lanes 2–6) or presence (lanes 7–11) of 60 μM *m*-AMSA. Lane 1, DNA standard; lanes 2–6 and 7–11, religation times of 0, 5, 10, 15, and 30 s, respectively.

It is important to ensure that all of the post-strand-passage DNA breakage generated in the presence of *m*-AMSA or etoposide was mediated solely by topoisomerase II. The data presented in Figure 5 demonstrate that this is the case. First, all DNA cleaved in the presence of drugs was covalently attached to topoisomerase II. Reaction mixtures had to be treated with proteinase K in order for cleaved products to be released (compare lane 2 with 3 and lane 5 with 6). The covalent linkage of enzyme to cleaved DNA is a hallmark of the topoisomerase II mediated reaction (Liu et al., 1983; Sander & Hsieh, 1983; Osheroff & Zechiedrich, 1987). Second, in the absence of the enzyme, no DNA cleavage was observed in reactions that contained APP(NH)P and either drug.

Inhibition of Topoisomerase II Mediated Post-Strand-Passage DNA Religation by Antineoplastic Drugs. Since antineoplastic drugs increase topoisomerase II mediated post-strand-passage DNA breakage, the effects of *m*-AMSA and etoposide on the enzyme's post-strand-passage DNA religation activity were determined. The heat-induced DNA religation assay was employed for these experiments. Assays were performed as described for Figure 3, except that antineoplastic drugs were added to reaction mixtures after post-strand-passage DNA cleavage/religation equilibria were established, but before samples were shifted from 30 to 55 °C. Reaction products from a typical assay carried out in the absence or presence of 60 μM *m*-AMSA are displayed in Figure 6. In this case, samples were subjected to electrophoresis in the absence of ethidium bromide so that the DNA topoisomers which resulted from enzyme-mediated strand passage could be visualized. Clearly, as determined by either the loss of linear DNA or the increase in covalently closed DNA topoisomers, *m*-AMSA inhibited the ability of topoisomerase II to mediate post-strand-passage religation. The transient rise in the levels of nicked DNA observed in Figure 6 confirms that nicked molecules are an obligatory kinetic intermediate in the enzyme's post-strand-passage religation of double-stranded breaks (i.e., linear DNA → nicked DNA → covalently closed DNA) (Fersht, 1985). At longer time points, levels of nicked molecules dropped at a rate comparable to that for the increase in covalently closed plasmid molecules.

Subsequent religation assays were subjected to electrophoresis in the presence of ethidium bromide in order to increase the mobility of partially relaxed DNA topoisomers (Maniatis et al., 1982) and facilitate the quantitation of linear molecules. Results for experiments which contained *m*-AMSA (60 μM) or etoposide (100 μM) are shown in Figure 7. As determined by the heat-induced reaction, both drugs stabilized the post-strand-passage DNA cleavage complex of *Drosophila* topoisomerase II primarily by decreasing the rate of religation. The

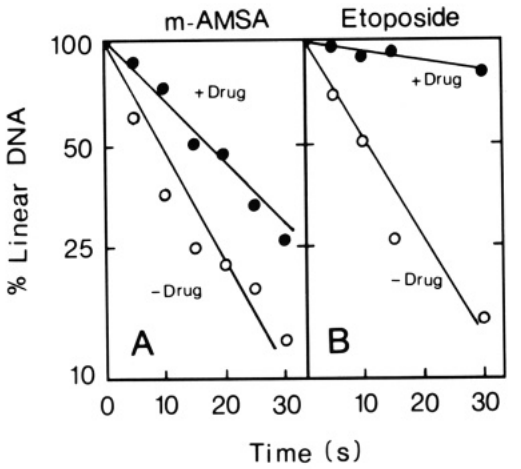

FIGURE 7: Effects of 60 μM *m*-AMSA (panel A) and 100 μM etoposide (panel B) on the ability of topoisomerase II to mediate post-strand-passage DNA religation at 55 °C. Religation was initiated by shifting assay mixtures from 30 to 55 °C as described under Experimental Procedures. Results are plotted in a semilogarithmic fashion as the loss of linear DNA versus time in the absence (open circles) or presence (closed circles) of drug. The percent linear DNA for each assay was arbitrarily set at 100% at time zero. Plots represent the average of three independent experiments.

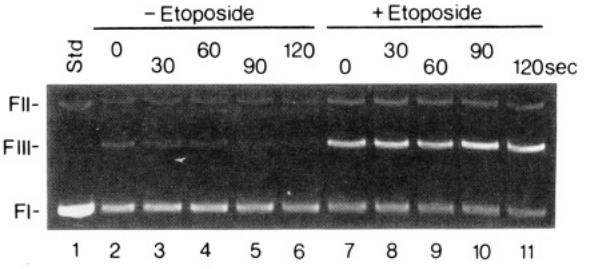

FIGURE 8: Effect of etoposide on the ability of topoisomerase II to mediate post-strand-passage DNA religation at 0 °C. Religation was initiated by shifting assay mixtures from 30 to 0 °C as described under Experimental Procedures. An agarose gel run in the presence of ethidium bromide (0.5 μg/mL) is shown. Assays were carried out in the absence (lanes 2–6) or presence (lanes 7–11) of 100 μM etoposide. Lane 1, DNA standard; lanes 2–6 and 7–11, religation times of 0, 30, 60, 90, and 120 s, respectively.

effects of etoposide on this reaction were more pronounced than those of *m*-AMSA. This is consistent with the increased ability of etopside to stabilize the enzyme's post-strand-passage DNA cleavage complex (see Figure 4).

In order to confirm the results of heat-induced religation assays, a second DNA religation protocol was employed. This second system takes advantage of the differential ability of topoisomerase II (as described above) to mediate DNA religation but not cleavage at 0 °C (Liu et al., 1983). Thus, as found for the heat-induced reaction, time-dependent DNA religation can also be induced by shifting reaction mixtures from 30 to 0 °C. Products of a typical assay carried out in the absence or presence of 100 μM etoposide are shown in Figure 8. In this case, samples were subjected to electrophoresis in an agarose gel which contained ethidium bromide. Results of experiments which examined the effects of *m*-AMSA (60 μM) or etoposide (100 μM) on cold-induced DNA religation are quantitated in Figure 9. These data confirm that antineoplastic drugs impair the ability of topoisomerase II to mediate DNA religation following strand passage.

Discussion

Topoisomerase II is the primary physiological target for several classes of antineoplastic drugs (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989), many of which are in wide use for the chemotherapeutic treatment of human cancers (Cassileth

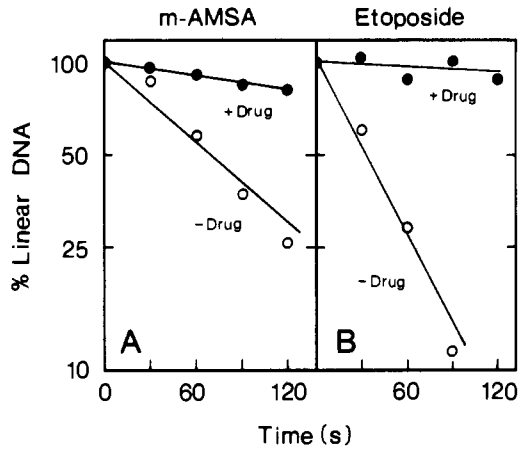

FIGURE 9: Effects of 60 μM *m*-AMSA (panel A) and 100 μM etoposide (panel B) on the ability of topoisomerase II to mediate post-strand-passage DNA religation at 0 °C. Religation was initiated by shifting assay mixtures from 30 to 0 °C as described under Experimental Procedures. Results are plotted in a semilogarithmic fashion as the loss of linear DNA versus time in the absence (open circles) or presence (closed circles) of drug. The percent linear DNA for each assay was arbitrarily set at 100% at time zero. Plots represent the average of three independent experiments.

& Gale, 1986; van Maanen et al., 1988; Fleming et al., 1989). The clinical efficacies of these agents correlate with their abilities to stabilize complexes between topoisomerase II and cleaved DNA (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). The enzyme establishes such DNA cleavage complexes both prior to and following its strand passage event (Osheroff, 1989a). Previous studies indicate that antineoplastic drugs stabilize the pre-strand-passage complex (Chen et al., 1984; Pommier et al., 1985; Osheroff, 1989b; Robinson & Osheroff, 1990) and at least *m*-AMSA and etoposide do so by inhibiting the ability of topoisomerase II to religate cleaved DNA (Osheroff, 1989b; Robinson & Osheroff, 1990). The present study demonstrates that these two structurally disparate drugs also stabilize the enzyme's post-strand-passage DNA cleavage complex and once again do so primarily by interfering with religation. These results strongly suggest that both the pre- and post-strand-passage DNA cleavage complexes of topoisomerase II serve as physiological targets for drug action.

Despite the mechanistic similarities discussed above, there are subtle differences in the actions of antineoplastic drugs before and after strand passage. On the one hand, the maximal effects of *m*-AMSA and etoposide on cleavage/religation require lower drug concentrations following strand passage. On the other, both drugs exhibit a decreased relative ability to stabilize the post-strand-passage complex. This latter observation may reflect the fact that in the absence of drugs the post-strand-passage DNA cleavage complex is already 3–5 times more stable than its pre-strand-passage counterpart. Several reports concerning the activities of drug-resistant topoisomerase II mutants also point to relationships between strand passage and drug action. First, a human type II enzyme selected for its resistance to *m*-AMSA shows cross-resistance to etoposide in the absence of ATP (the cofactor required for DNA strand passage) but sensitivity to the drug in the presence of the nucleoside triphosphate (Zwelling et al., 1989). Second, a human topoisomerase II selected for its resistance to teniposide requires approximately 8 times more ATP for catalytic activity than does the native enzyme (Danks et al., 1989). Finally, the mutation in an *m*-AMSA-resistant bacteriophage T4 topoisomerase II maps to the subunit of the enzyme which interacts with ATP (Huff et al., 1989).

In order to define the physiological actions of topoisomerase II targeted drugs, it is necessary to elucidate their effects on each step of the enzyme's catalytic cycle. Since the ATP-promoted strand-passage activity of topoisomerase II is essential for the viability of eukaryotic cells (Vosberg, 1985; Wang, 1985; Osheroff, 1989a), it is especially important to characterize the effects of strand passage on drug action. The finding that antineoplastic agents stabilize both the enzyme's pre- and post-strand-passage DNA cleavage complexes provides a clearer understanding of the mechanism by which these drugs alter the activity of topoisomerase II.

ACKNOWLEDGMENTS

We thank L. Smith and C. Brewer for their assistance in preparing the topoisomerase II and pBR322 plasmid DNA employed for this study, A. H. Corbett for her critical reading of the manuscript, and S. Heaver for her conscientious preparation of the manuscript.

REFERENCES

Berrios, M., Osheroff, N., & Fisher, P. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4142–4146.

Bolivar, F., Rodriguez, R., Greene, P. J., Betlach, M., Heyneker, H. L., Boyer, H. W., Crosa, J., & Falkow, S. (1977) *Gene 2*, 95–113.

Cassileth, P. A., & Gale, R. P. (1986) *Leukemia Res. 10*, 1257–1265.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M., & Liu, L. F. (1984) *J. Biol. Chem. 21*, 13560–13566.

Danks, M. K., Schmidt, C. A., Deneka, D. A., & Beck, W. T. (1989) *Cancer Commun. 1*, 101–109.

DiNardo, S., Voelkel, K., & Sternglanz, R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2616–2620.

Earnshaw, W. C., & Heck, M. M. S. (1985) *J. Cell Biol. 100*, 1716–1725.

Earnshaw, W. C., Halligan, B., Cooke, C. A., Heck, M. M. S., & Liu, L. F. (1985) *J. Cell Biol. 100*, 1706–1715.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman, New York.

Fleming, R. A., Miller, A. A., & Stewart, C. F. (1989) *Clin. Pharmacol. 8*, 274–293.

Gale, K. C., & Osheroff, N. (1990) *Biochemistry 29*, 9538–9545.

Gasser, S. M., & Laemmli, U. K. (1986) *EMBO J. 5*, 511–518.

Gasser, S. M., Laroche, T., Falquet, J., Boy De La Tour, E., & Laemmli, U. K. (1986) *J. Mol. Biol. 188*, 613–629.

Glisson, B. S., & Ross, W. E. (1987) *Pharmacol. Ther. 32*, 89–106.

Goto, T., & Wang, J. C. (1984) *Cell 36*, 1073–1080.

Holm, C., Goto, T., Wang, J. C., & Botstein, D. (1985) *Cell 41*, 553–563.

Hsiang, Y.-H., & Liu, L. F. (1989) *J. Biol. Chem. 264*, 9713–9715.

Hsiang, Y.-H., Jiang, J. B., & Liu, L. F. (1989) *Mol. Pharmacol. 36*, 371–376.

Huff, A. C., Leatherwood, J. K., & Kreuzer, K. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1307–1311.

Liu, L. F. (1989) *Annu. Rev. Biochem. 58*, 351–375.

Liu, L. F., Rowe, T. C., Yang, L., Tewey, K. M., & Chen, G. L. (1983) *J. Biol. Chem. 258*, 15365–15370.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning—A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nelson, E. M., Tewey, K. M., & Liu, L. F. (1984) *Proc. Natl. Sci. U.S.A. 81*, 1361–1365.

Osheroff, N. (1986) *J. Biol. Chem. 261*, 9944–9950.

Osheroff, N. (1989a) *Pharmacol. Ther. 41*, 223-241.
Osheroff, N. (1989b) *Biochemistry 28*, 6157-6160.
Osheroff, N., & Zechiedrich, E. L. (1987) *Biochemistry 26*, 4303-4309.
Osheroff, N., Shelton, E. R., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9530-9535.
Pommier, Y., Minford, J. K., Schwartz, R. E., Zwelling, L. A., & Kohn, K. W. (1985) *Biochemistry 24*, 6410-6416.
Robinson, M. J., & Osheroff, N. (1990) *Biochemistry 29*, 2511-2515.
Sander, M., & Hsieh, T.-S. (1983) *J. Biol. Chem. 258*, 8421-8428.
Sander, M., Hsieh, T.-S., Udvardy, A., & Schedl, P. (1987) *J. Mol. Biol. 194*, 219-229.
Shelton, E. R., Osheroff, N., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9530-9535.
Tewey, K. M., Chen, G. L., Nelson, E. M., & Liu, L. F. (1984) *J. Biol. Chem. 259*, 9182-9187.
Uemura, T., & Yanagida, M. (1984) *EMBO J. 3*, 1737-1744.
Uemura, T., & Yanagida, M. (1986) *EMBO J. 5*, 1003-1010.
Uemura, T., Ohkura, H., Adachi, Y., Morino, K., Shiozaki, K., & Yanagida, M. (1987) *Cell 50*, 917-925.
van Maanen, J. M. S., Retel, J., de Vries, J., & Pinedo, H. M. (1988) *J. Natl. Cancer Inst. 80*, 1526-1533.
Vosberg, H.-P. (1985) *Curr. Top. Microbiol. Immunol. 114*, 19-102.
Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665-697.
Yang, L., Rowe, T. C., & Liu, L. F. (1985) *Cancer Res. 45*, 5872-5876.
Zechiedrich, E. L., Christiansen, K., Andersen, A. H., Westergaard, O., & Osheroff, N. (1989) *Biochemistry 28*, 6229-6236.
Zwelling, L. A. (1985) *Cancer Metastasis Rev. 4*, 263-276.
Zwelling, L. A., Hinds, M., Chan, D., Mayes, J., Sie, K. L., Parker, E., Silberman, L., Radcliffe, A., Beran, M., & Blick, M. (1989) *J. Biol. Chem. 264*, 16411-16420.

Kinetic and Equilibrium Analysis of a Threading Intercalation Mode: DNA Sequence and Ion Effects†

Farial A. Tanious, Shau-Fong Yen,‡ and W. David Wilson*
Department of Chemistry and Laboratory for Chemical and Biological Sciences, Georgia State University, Atlanta, Georgia 30303

Received September 6, 1990; Revised Manuscript Received November 19, 1990

ABSTRACT: The interaction of a symmetric naphthalene diimide with alkylamino substituents at each imide position was investigated with the alternating sequence polymers, poly[d(A-T)]$_2$ and poly[d(G-C)]$_2$. Spectrophotometric binding studies indicate strong binding of the diimide to both sequences although the GC binding constant is 20-25 times larger than the AT binding constant. Analysis of the effects of salt concentration on the binding equilibria shows that the diimide forms two ion pairs in its complex with both polymers as expected for a simple dication. Stopped-flow kinetics experiments demonstrate that the diimide both associates and dissociates from DNA more slowly than classical intercalators with similar binding constants. Analysis of salt concentration effects on dissociation kinetics rate constants (k_d) reveals that slopes in log k_d versus log [Na^+] plots are only approximately half the value obtained for classical dicationic intercalators that have both charged groups in the same groove. These kinetics results support a threading intercalation model, with one charged diimide substituent in each of the DNA grooves rather than with both side chains in the same groove, for the diimide complex with DNA. In the rate-determining step of the mechanism for dissociation of a threading complex only one ion pair is broken; the free side chain can then slide between base pairs to put both diimide side chains in the same groove, and this is followed by rapid full dissociation of the diimide. This sequential release of ion pairs makes the dissociation slope for dicationic threading intercalators more similar to the slope for classical monocationic intercalating ligands. Kinetics studies, thus, provide a very clear method for distinguishing classical from threading intercalators. Similar experiments can also distinguish intercalation from groove binding modes.

Simple intercalators, such as proflavin, bind to DNA with essentially all of their aromatic system (e.g., their entire molecular structure) inserted between the base pairs that form the top and bottom of the intercalation site (Waring, 1981; Neidle & Abraham, 1984; Wilson, 1990). Most natural and synthetic intercalators, however, have substituents of varying chemical nature, steric bulk, and charge which lie in one of the DNA grooves after insertion of the planar aromatic system of the intercalator between base pairs. Detailed crystallographic and NMR studies have shown that many well-characterized intercalators such as ethidium (Jain et al., 1977; Tsai et al., 1977), actinomycin (Sobell & Jain, 1972; Scott et al., 1988), and daunomycin (Wang et al., 1987) intercalate with DNA such that their bulky substituents are in the minor groove. Lower resolution experiments (binding, hydrodynamic, etc.) coupled with modeling studies have suggested that many acridine intercalators, such as the anticancer drug amsacrine, also bind with their bulky substituents in the minor groove (Denny et al., 1983; Abraham et al., 1988).

Several recently characterized intercalators, however, form DNA complexes with bulky and/or charged substituents in the major groove of the double helix. A group of unfused aromatic intercalators, which are amplifiers of the bleomycin-catalyzed cleavage of DNA, bind to DNA in such a major groove complex (Wilson et al., 1988, 1989b). Some aromatic

† This work was supported by Grant NIH-NIAID AI-27196.
‡ Present address: Ciba Vision Corp., Atlanta, GA 30360.

0006-2960/91/0430-1813$02.50/0