Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1787–1791, April 1983
Biochemistry

Inhibition of *Micrococcus luteus* DNA topoisomerase I by UV photoproducts

(plasmid pAT153/gel electrophoresis/pyrimidine dimers)

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Communicated by Richard B. Setlow, December 3, 1982

ABSTRACT **The activity of *Micrococcus luteus* DNA topoisomerase I on UV-irradiated supercoiled DNA was studied under either processive or distributive reaction conditions. Changes in DNA structure caused by UV irradiation reduce the rate of DNA relaxation at very low concentration of photoproducts. Under processive conditions the inhibition of the topoisomerase I by photoproducts can be quantitated by measuring the amount of substrate left in the replicative form I band. The mode of action of DNA topoisomerase I was affected by the presence of photoproducts in the DNA substrate, although the ability of the enzyme to form a covalent complex with UV-irradiated supercoiled DNA was not changed. The inhibition of topoisomerase I by UV photoproducts has been compared to the effects of single-stranded DNA and UV-irradiated duplex linear DNA on the enzyme, and the results suggest that the inhibition by photoproducts is caused by changes in the conformation of the supercoil. Our findings indicate the possibility that DNA topoisomerase I plays a role in repair.**

The topological state of DNA and the enzymes that control this state play a crucial role in determining the function of DNA in cells. A class of enzymes called DNA topoisomerases, which promote the conversion of one topological isomer to another through the concerted breakage and rejoining of phosphodiester bonds, is thought to regulate the superhelical conformation of DNA within the cell. Topoisomerases are ubiquitous enzymes that have been demonstrated in a variety of prokaryotic and eukaryotic species. Two types of DNA topoisomerases have been detected. Type I topoisomerases catalyze the conversion of superhelical DNA to the energetically favored relaxed form without a nucleotide cofactor, whereas type II enzymes require ATP for their action on DNA regardless of whether they relax or supercoil DNA (1, 2).

Studies carried out with bacterial mutants defective in DNA topoisomerases have indicated that the three-dimensional structure of the bacterial chromosome is important not only for the processes of DNA replication, transcription, and genetic recombination but also for the processes of DNA repair. In fact these mutants showed an increased sensitivity to the killing effect of UV radiation (3–7). Furthermore, novobiocin and nalidixic acid, two specific inhibitors of type II topoisomerase, affected to different degrees the amount of UV-stimulated repair synthesis in *Escherichia coli* (8–10) and inhibited the recovery of UV-irradiated nonreplicating λ phage (11).

The aim of our work was to study the action of DNA topoisomerases on UV-irradiated substrates to obtain information on the relevance of DNA three-dimensional structure in repair processes as well as on the interaction between proteins and damaged DNA. It has been shown that low concentrations of UV photoproducts in supercoiled DNA generate changes that are not identical with local denaturation (12–14). These changes, consisting of an unwinding of the double helix of less than one base pair per pyrimidine dimer, have been detected after agarose gel electrophoresis of single topological DNA species (15).

We have already shown that UV-induced modifications in the three-dimensional structure of DNA have no effect on the supercoiling activity of *Bacillus subtilis* DNA topoisomerase II (16). In this communication we report on the effects of UV irradiation of supercoiled DNA on the relaxing activity of *Micrococcus luteus* DNA topoisomerase I.

MATERIALS AND METHODS

Preparation of DNAs, UV Irradiation, and Pyrimidine Dimer Titration. pAT153, a plasmid of 3,657 base pairs derived from pBR322 (17), was chosen as substrate for DNA topoisomerase I because of its high copy number. Naturally supercoiled pAT153 DNA was obtained from stationary-phase *E. coli* HB101 (18) grown in L broth containing tetracycline at 10 μg/ml. Plasmid replicative form I (RFI) DNA was isolated by alkali lysis and CsCl/ethidium bromide density gradient sedimentation (19) followed by sedimentation in a neutral sucrose gradient and was stored at −14°C in 10 mM Tris·HCl, pH 8.0/0.1 mM NaEDTA. Phage M13 DNA was prepared from the viral particles according to Fidanian and Ray (20). Plasmid pRLM4, a derivative of pMB9, digested with *Sma* I to yield fragments of 2,600 and 3,400 base pairs, was a gift of R. McMacken. DNA was irradiated with UV light generated by a 15-W low-pressure mercury vapor germicidal lamp (254-nm wavelength; Philips TUV) at the indicated doses at the rate of 1.1 $J \cdot m^{-2} \cdot s^{-1}$. Pyrimidine dimer titration was performed as described (12) by measuring the number of nicks introduced into UV-irradiated supercoiled pAT153 DNA by *M. luteus* pyrimidine dimer endonuclease. Intact molecules (RFI) were separated from the nicked ones (RFII) by electrophoresis on agarose gels, and the relative amounts of the two forms were determined by scanning the negatives of gel photographs. The number of nicks per molecule was calculated by Poisson analysis. The same type of analysis was used to measure the DNA topoisomerase I–DNA complex formed in the absence of Mg^{2+}.

Enzymes. *M. luteus* pyrimidine dimer endonuclease was purified through the CM-cellulose step according to Haseltine *et al.* (21). *M. luteus* DNA topoisomerase I was purified to homogeneity as described by Kung and Wang (22). Protein concentration, as measured by the technique of Bradford (23), was 0.27 mg/ml. This enzyme preparation had a specific activity of 6.5×10^5 units/mg. In this work one unit of DNA topoisomerase I is defined as the amount of enzyme that con-

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Abbreviation: RF, replicative form.

verts 50% of RFI DNA to other forms under the assay conditions described below. However, because the activity of the *M. luteus* enzyme changes under the different experimental conditions used in this work we prefer to express the amount of enzyme used in ng instead of units.

DNA Topoisomerase I Assay Conditions. The 25-μl assay mixture contained 0.05 M NaHepes at pH 7.6, 0.001 M Na_3EDTA, 0.005 M $MgCl_2$, 0.10–0.20 pmol of supercoiled pAT153 DNA substrate, and 1–5 ng of enzyme. After incubation for 10 min at 30°C the reaction was terminated by the addition of 5 μl of tracking dye stock solution containing bromophenol blue at 0.25 mg/ml, 50% (vol/vol) glycerol, and $NaDodSO_4$ at 5 mg/ml. DNA samples were loaded on a 1% agarose (Bio-Rad) slab gel (143 × 134 × 4 mm) and electrophoresis was carried out at 25 V for 15 hr at room temperature. The electrophoresis buffer was 0.05 M Tris base, 0.38 M glycine, and 0.002 M NaEDTA. Gels were stained for 30 min with ethidium bromide at 1 μg/ml and DNA was visualized on a Chromato-Vue transilluminator C-61 box (Ultraviolet Products, San Gabriel, CA) and photographed on Polaroid type P55 films through a yellow filter. The negatives were traced with a Beckman DU-8 computerized gel scanning system to calculate the proportion of the RFI DNA as described (12).

RESULTS

Inhibition of DNA Topoisomerase I by UV Photoproducts. To study the influence of UV photoproducts on the activity of *M. luteus* DNA topoisomerase I we have used different reaction conditions because type I DNA topoisomerases are known to act in a processive or a distributive way depending on the salt concentrations (22, 24). Agarose gel analysis of reaction products was used to discriminate between these two different modes of action. Naturally supercoiled substrate DNA is a mixture of highly superhelical topological isomers that migrate together, forming the RFI band. Under the reaction and electrophoresis conditions utilized, the final isomer distribution for the completely relaxed products was found to center at the nicked form of circular DNA (RFII) (Fig. 1, lanes e and l). Topological DNA species with an intermediate number of turns were found migrating between these two extremes. When 70 mM potassium phosphate buffer was used, DNA topoisomerase I acted in a distributive way as suggested by the distribution of the intermediates of the reaction. Under these conditions all RFI substrate disappeared before any significant amount of final product could be observed. The population of partially relaxed molecules appeared in a Gaussian distribution centering at different positions with respect to the position of nicked DNA as a function of enzyme concentration (Fig. 1, lanes f–l). Therefore all the DNA molecules in the presence of a smaller number of protein molecules appeared affected. We have found that not only lowering the concentration of phosphate buffer (22) but also substituting Na Hepes for it caused the action of DNA topoisomerase I to become processive. In fact, substantial amounts of final product were formed while part of the substrate still remained unchanged in the RFI position. The distribution of intermediate isomers did not change (Fig. 1, lanes b–d).

The effects of UV light damage on the activity of DNA topoisomerase I when assayed under processive reaction conditions are shown in Fig. 2. Increasing exposure of DNA to UV light causes progressive reduction of the amount of relaxed products. These results indicate an inhibition of DNA topoisomerase I activity. The inhibition is particularly evident from the simultaneous increase in intensity of the RFI band. We have quantified this inhibition by measuring the increase in the relative amount of DNA present in the RFI position as the total amount of DNA per lane remained unchanged. The inhibition of relaxation measured in this way could be observed even at doses producing less than an average of one dimer per molecule. The inhibition is independent of the DNA-to-protein molar ratio between 2 and 8 (Fig. 3).

The consequences of UV damage for the activity of DNA topoisomerase I under distributive conditions are shown in Fig. 4*A* (lanes a–h). Two main effects can be observed. At low doses (Fig. 4, lanes b and c) the center of the distribution is shifted toward the RFI position as in the presence of single-stranded DNA (Fig. 4*A*, lanes j–o), a known competitive inhibitor of DNA topoisomerase I (22, 25), suggesting an inhibition. At higher doses of UV light the distribution is no longer Gaussian, the intensity of each band tends to become more uniform, and more relaxed forms start to appear while

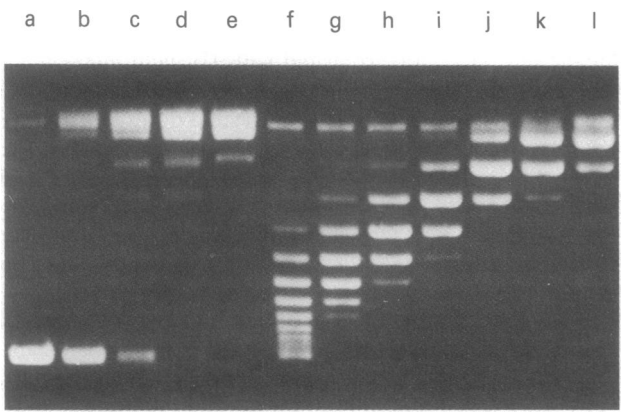

FIG. 1. Effect of different buffers on the processivity of *M. luteus* DNA topoisomerase I by agarose gel analysis of reaction products. Reaction mixtures (25 μl each) contained 0.005 M $MgCl_2$, 0.001 M EDTA, and 0.20 pmol of superhelical pAT153 DNA. Processive or distributive mode of action was obtained by using 0.05 M NaHepes (pH 8.0) (lanes b–e) or 70 mM potassium phosphate (pH 8.0) (lanes f–l). The reaction mixtures were incubated for 10 min at 30°C with the following amounts of DNA topoisomerase I: b, 2.1 ng; c and f, 4.2 ng; d and g, 8.4 ng; e and h, 17 ng; i, 33 ng; j, 66 ng; k, 135 ng; l, 270 ng; a, enzyme omitted.

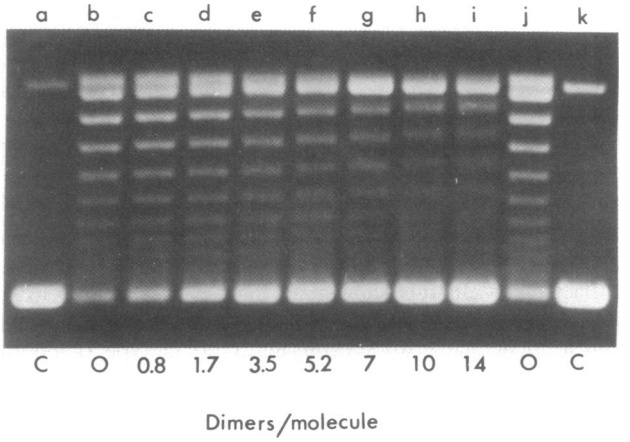

FIG. 2. Effect of 254-nm light on DNA topoisomerase I acting processively. Reaction mixtures (25 μl each) containing 0.05 M NaHepes (pH 7.6), 0.005 M $MgCl_2$, 0.001 M EDTA, 5.5 ng of *M. luteus* DNA topoisomerase I, and 0.20 pmol of superhelical pAT153 DNA were incubated at 30°C for 10 min and reaction products were analyzed on an agarose gel. DNA samples were irradiated with increasing doses of 254-nm light and the dimer content was calculated from the number of sites sensitive to the *M. luteus* pyrimidine dimer endonuclease. C, control with no enzyme.

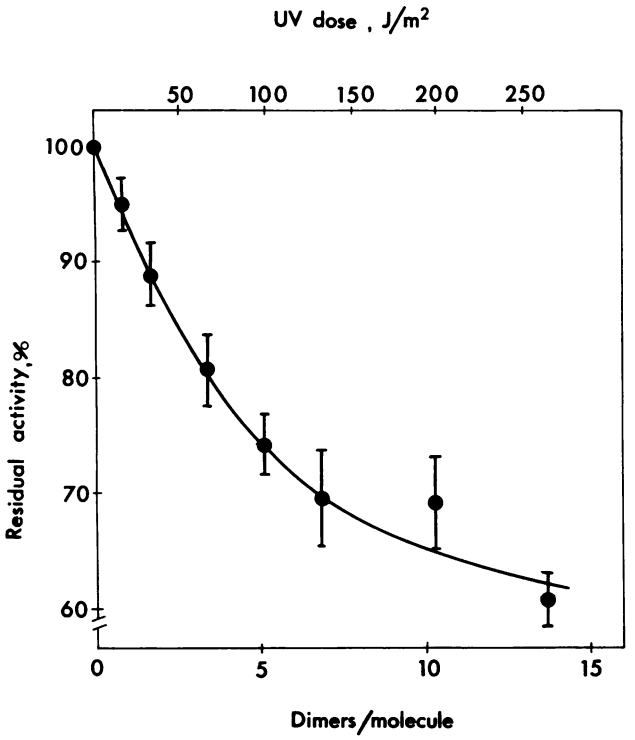

FIG. 3. Inhibition curve by 254-nm light of DNA topoisomerase I acting processively. Reaction mixtures were as described for Fig. 2 except for the amount of DNA topoisomerase I, which varied between 2 and 8 ng per assay. The activity was evaluated by densitometric scanning of negatives of gel photographs at 700 nm on a Beckman DU-8 gel scanner; 100% activity is defined as the amount of relaxed substrate with a mobility lower than the RFI band in unirradiated samples. Each plotted value is the average of five independent experiments in which the ratio of DNA to enzyme varied by a factor of 4. Bars indicate SD.

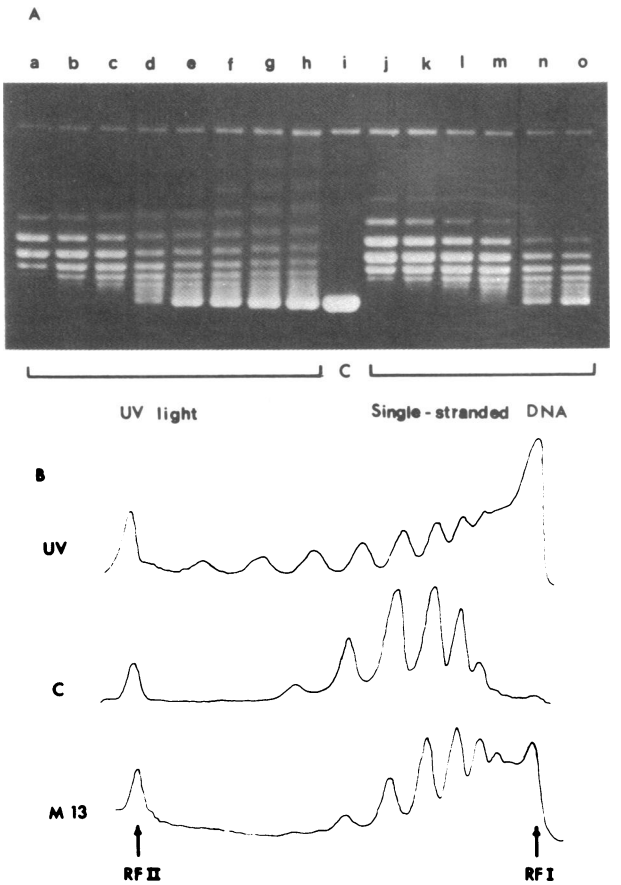

FIG. 4. (*A*) Inhibition by 254-nm light and by M13 phage DNA of DNA topoisomerase I acting nonprocessively. Reaction mixture was as described for Fig. 1, lane g. Dimer content per molecule was a, 0; b, 0.8; c, 1.7; d, 3.5; e, 5.2; f, 7.0; g, 10; h, 14. Single-stranded M13 DNA content was j, none; k, 0.08 μM; l, 0.16 μM; m, 0.32 μM; n, 0.97 μM; o, 1.30 μM. Lane i, no enzyme. (*B*) Densitometric traces of lanes g, j, and n.

a consistent amount of substrate remains in the RFI position. The loss of the Gaussian distribution has made difficult any quantitative comparison between the inhibition by single-stranded DNA and UV light under these conditions. The possibility that the appearance of final products could be ascribed to a selective action of DNA topoisomerase I on molecules with damage content different from the average has been excluded because of the position of each single topoisomer band, a very reliable parameter of the damage load. We have in fact shown previously that UV photodamage reduces the mobility of single topoisomers on agarose gel as a linear function of the amount of UV photoproducts (12). The appearance of final products while a considerable amount of substrate remained in the RFI position, coupled to the loss of the Gaussian distribution not due to the action of DNA topoisomerase I on a selected population of molecules, suggests a modification in the mode of action of the enzyme to a more processive one.

A quantitative comparison was possible under processive conditions of the reaction because, judging from the distribution of reaction products, there was no observable change in the mode of action of DNA topoisomerase I after UV irradiation (Fig. 2). The results obtained are presented in Fig. 5. It can be seen that increasing amounts of M13 DNA or UV irradiation cause a linear decrease in the reaction rate. This decrease corresponded to 10% reduction in the reaction rate every 10 pmol (nucleotide phosphorus) of single-stranded DNA per assay. The same reduction could be obtained with a UV dose corresponding to 0.41 pmol of dimer per assay. These data show that UV photoproducts can be considered a more effective inhibitor because the inhibition by a UV dose generating one dimer per molecule is equivalent to the presence of 24 bases as single-stranded DNA per molecule of substrate.

We have also tested the possibility that photoproducts on DNAs other than the supercoiled substrate could act as competitive inhibitors. DNA topoisomerase I was assayed under processive reaction condition in the presence of either single-stranded M13 phage DNA or blunt-ended restriction fragments of pRLM4 carrying amounts of photodamage as the substrate of the experiment shown in Fig. 2. The lack of inhibition observed in both cases indicates that neither the photoproducts nor the induced local denatured regions they are supposed to generate in linear duplex DNA (26–28) could act as inhibitors.

Interaction Between UV-Irradiated Supercoiled DNA and DNA Topoisomerase I. In the absence of Mg^{2+}, DNA topoisomerase I forms a stable complex with highly supercoiled or single-stranded DNA (29, 30). Addition of $NaDodSO_4$ or proteinase K to such a complex causes the formation of a single chain scission. It is supposed that this DNA–protein complex represents an intermediate in the topological isomerization of supercoiled DNA. We have therefore explored the possibility that formation of this reaction intermediate is impeded when the DNA is irradiated with UV light. The amount of complex is evaluated by measuring, after electrophoresis on an agarose gel, the amount of RFII produced by exposure of RFI–protein complex to $NaDodSO_4$/proteinase K. We have found that up to a UV dose responsible for 40% inhibition there is no detectable reduction in the amount of

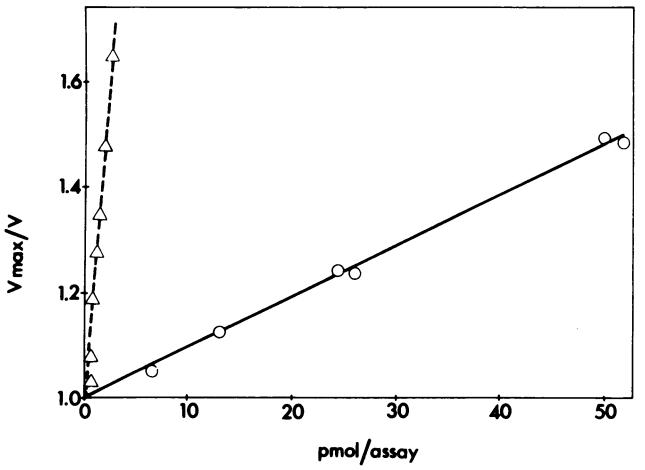

FIG. 5. Single-stranded DNA and pyrimidine dimer inhibition of DNA topoisomerase I acting processively. Reaction mixtures as in Fig. 2 were supplemented with increasing concentrations of single-stranded circular M13 phage DNA (measured as pmol of nucleotide) or superhelical DNA irradiated with increasing UV doses (measured as pmol of dimers). The inhibition was measured as described for Fig. 2. The reduction of reaction rate by single-stranded DNA (○) was compared with that generated by UV light (△) assuming that pyrimidine dimers are the photoproducts responsible for the inhibition.

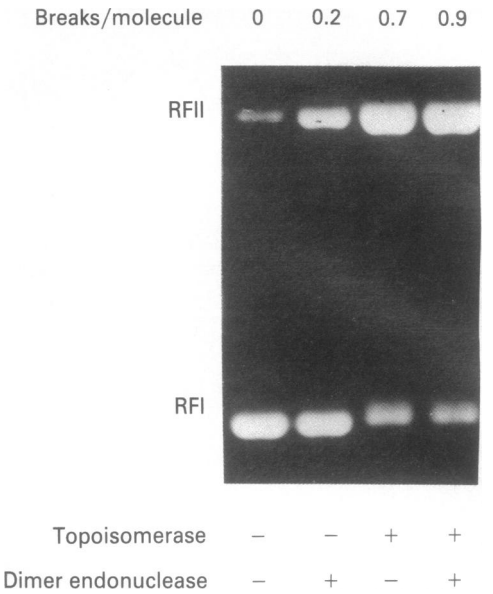

FIG. 6. Cleavage of DNA backbone upon $NaDodSO_4$ treatment and pyrimidine dimer endonuclease treatment of the complex between DNA topoisomerase I and UV-irradiated pAT153 DNA. Reaction mixtures (25 μl each) containing 50 mM Tris·HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 0.10 pmol of superhelical pAT153 DNA, and 0.54 μg of homogeneous DNA topoisomerase I incubated at 37°C for 15 min. Saturating amounts of *M. luteus* pyrimidine dimer endonuclease were then added where indicated and the mixture was further incubated for 10 min at 37°C. Five microliters of glycerol tracking dye solution containing proteinase K at 0.2 mg/ml was added and the mixture was incubated at 37°C for another 60 min. The samples were then loaded on 1% agarose slab gel and run for 15 hr at 20 V.

complex formed (data not shown). However, with our assay conditions we cannot investigate the possibility of whether UV damage may instead increase the binding.

The UV doses used in all these experiments are low enough to justify the assumption that pyrimidine dimers are the main photoproduct of 254-nm light responsible for the inhibition (31). We have therefore investigated the possibility of a direct interaction between DNA topoisomerase I and dimers, namely the specific binding of the enzyme at the dimer level. We have tested this possibility by measuring the accessibility of pyrimidine dimers to the action of *M. luteus* dimer-specific endonuclease on a complex of DNA topoisomerase I with UV-irradiated DNA. The results shown in Fig. 6 indicate that the binding is not influenced by the presence of dimers, because the nicking due to the action of *M. luteus* endonuclease on the DNA–protein complex is additive to the nicking induced by $NaDodSO_4$/proteinase K.

DISCUSSION

Our results show that conformational changes due to the formation of UV photodamage influence the mode of action and inhibit the relaxing activity of *M. luteus* DNA topoisomerase I. Because the inhibition can be detected at doses producing less than an average of one dimer per molecule of 3,657 base pairs, the effect of other photoproducts can be considered negligible. Contribution of crosslinks to the inhibitory effect can also be excluded because even at the highest dose used their formation may be considered insignificant (32). It is therefore possible to assign this inhibition primarily to pyrimidine dimer formation.

The reaction rate of the DNA topoisomerase I is highly dependent on the sense and degree of superhelicity of DNA substrate and the reaction is strongly inhibited by limited amounts of single-stranded DNA. Comparison between the inhibition by increasing amounts of single-stranded M13 phage DNA and by pyrimidine dimers shows that one dimer per molecule is equivalent to the effect of 24 single-stranded nucleotides. It also shows that UV damage alters the mode of action of the enzyme. In addition we have shown that UV-photodamaged DNA other than the supercoiled substrate does not act as competitive inhibitor, as single-stranded DNA is supposed to do (30). By using irradiated linear duplex DNA we have also obtained a strong indication that changes in the secondary structure of DNA due to pyrimidine dimers are not responsible for the inhibition. In previous work we have calculated an unwinding angle of −14° or less per dimer, corresponding to an unpairing of less than one base pair per dimer (11, 14). The present data can therefore be considered a further indication that the unwinding of the DNA helix after irradiation of a supercoiled substrate causes a change in helix configuration without a significant disruption of base pairing.

DNA topoisomerase I, in its nicking-closing activity, involves the formation of a transient covalent DNA–protein bond. We have shown that the formation of this DNA–protein complex is not reduced by UV irradiation and it does not occur in the immediate vicinity of a dimer, suggesting that the inhibition must take place in one of the subsequent steps of the reaction. Therefore the more reasonable explanation for the inhibition seems to be the introduction of a torsional strain in the helix containing the dimer. Such torsional strain can have different consequences, depending on whether the enzyme is bound to the helix containing the dimer or to the opposite one. In the former case one might expect a modification in the binding of the enzyme to DNA as suggested by the tendency of the reaction to shift from nonprocessive to processive. In the latter, one might expect a slowdown of the diffusion of the helix through the DNA–protein complex, resulting in the inhibition of the reaction. The distance of the dimer from the complex could influence the extent of the inhibition.

If this hypothesis is correct it is possible to anticipate that agents causing similar torsional strains in supercoiled DNA will act as inhibitors of DNA topoisomerase I. An alternative possible explanation for the observed phenomena is that the

active binding of the enzyme is directed by secondary or higher structural features of the DNA that are disrupted by dimers.

These observations can provide some insight into the possible role of superhelicity of the chromosome in the repair processes. The observation that one of the pleiotropic effects in an *E. coli* mutant deficient in topoisomerase I consists of increased sensitivity to the lethal effects of UV supports this possibility (5). Enhancement in transcription of inducible operons has also been observed in this mutant; therefore an indirect effect on the modulation of inducible repair can be postulated. Such a possibility is confirmed by the finding that an analogous mutant in *Salmonella typhimurium* is not only UV sensitive but also deficient in UV-induced mutagenesis (6).

The negative superhelicity of the chromosome could to some extent direct repair processes. In fact, the modified relaxation of *in vivo* irradiated superhelical DNA might leave the DNA in a reactive state important for subsequent steps in repair. It is reasonable to envisage that enzymic incision systems with a broad damage specificity, such as the *uvr* system of *E. coli*, might recognize, in addition to the chemical lesion in DNA, a more general structural alteration of the superhelicity (e.g., untwisting). On the contrary, the damage-specific endonucleases are known to be insensitive to DNA structure (33). The recent observation that a mammalian DNA repair endonuclease with broad specificity is active only on supercoiled DNA (34) supports this possibility. It might therefore be possible that superhelicity is an important factor in many DNA repair systems.

The authors thank Dr. Charles Radding for helpful discussions. The technical assistance of Mrs. Maria T. Chiesa is gratefully acknowledged. This work was supported by the Progetto Finalizzato of the Consiglio Nazionale delle Ricerche, Controllo della crescita neoplastica.

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