Volume 8 Number 24 1980 Nucleic Acids Research

Breakage of single-stranded DNA by rat liver nicking-closing enzyme with the formation of a DNA-enzyme complex

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Received 25 August 1980

ABSTRACT

The DNA nicking-closing enzyme (type I topoisomerase) from rat liver nuclei breaks single-stranded DNA. The broken strand contains a 5'-hydroxyl and tightly bound protein. The stability of this protein-DNA complex to high salt, alkali and detergent suggests a covalent linkage between the DNA and the enzyme. The observed breakage of single-stranded DNA occurs at neutral pH prior to treatment with alkali or detergent, indicating that the breakage may be the result of an interrupted nicking and closing cycle. The resulting covalent complex could represent a reaction intermediate in the overall nicking-closing reaction.

INTRODUCTION

Enzymes that affect DNA topology (DNA topoisomerases) were first discovered and assayed by their ability to catalyze the removal of negative superhelical turns from closed circular duplex DNA (1,2; for reviews see 3,4). To account for this reaction it was postulated that the enzymes act by introducing a transient single-strand break (nick) into duplex DNA (1,2). Here we will use the term nicking-closing enzyme to refer to those topoisomerases for which the available evidence indicates that DNA relaxation occurs by the introduction of transient nicks (3). Such topoisomerases belong to the type I class as defined by Liu et al. (5). Recently it has been found that some DNA topoisomerases act via a double-strand break (5,6,7).

In addition to the untwisting reaction observed with superhelical closed duplex DNA, reactions involving single-stranded DNA and one or more of the purified nicking-closing enzymes have been described. The formation of covalently closed duplex DNA from annealed complementary single-stranded circles is catalyzed by the rat liver enzyme (8). The *E. coli* nicking-closing enzyme (ω-protein), in addition to carrying out the above reaction (9), has been shown to have the property of catalyzing the introduction and removal of knots from single-stranded DNA circles (10).

Since the nicking and resealing of DNA is carried out by a single protein with no requirement for energy donating co-factors, it was suggested that the energy for resealing the nick may be stored in a covalent linkage between the enzyme and DNA (1,2). Complexes of nicked DNA and tightly associated protein have been detected in reactions with topoisomerases (6,11,12, 13,14). For the rat liver nicking-closing enzyme a nicked form of DNA with covalently attached protein can be isolated from reactions in which duplex DNA is the substrate by stopping the reactions with either alkali, low pH (11,12, 15) or SDS. Keller et al. (16) found a similar form using a nicking-closing enzyme purified from KB cells after stopping the reaction with alkali. Covalent linkage between protein and broken DNA strands has been detected with ω-protein (13,14) and DNA gyrase (6) from E. coli. The ω-protein requires Mg^{++} for nicking-closing activity on duplex DNA (1). In its absence, ω-protein and single-stranded DNA or negatively superhelical closed duplex DNA form a salt stable complex that is induced to form a covalently linked nicked DNA-protein complex by treatment with alkali or SDS (13,14). Incubation of DNA gyrase and duplex DNA in the presence of the gyrase inhibitor oxolinic acid followed by treatment with SDS results in a double-strand break with protein covalently attached to the end of each broken strand (6).

We report here that single-stranded DNA (ssDNA) is broken by the rat liver nicking-closing enzyme. Strand breakage can be detected prior to addition of denaturing agents or proteases and results in bound protein and a free 5'-hydroxyl.

MATERIALS AND METHODS

General. The sources for reagents and materials have been given (17, 18). The procedure for the preparation of ^{3}H-labeled SV40 DNA has been previously described (17). Bacteriophage ØX174am3 and bacterial strains E. coli HF4704 Su^{+}, thy^{-}, $ØX^{S}$ and E. coli HF4704 Su^{-}, her^{-}, thy^{-}, $ØX^{S}$ were obtained from T. Kunkel and L. Loeb (19). [^{3}H] thymidine and [$\gamma\ ^{32}P$] ATP were purchased from New England Nuclear Corporation. Soluene 350 was purchased from Packard. Seakem Agarose (M.E.) was purchased from Marine Colloids.

Preparation of ØX174am3 viral DNA. Isolation of the phage particles was essentially as described by Kunkel and Loeb (19). The isolated phage were banded twice in CsCl and phenol extracted as described (19). After ether extraction and concentration the DNA was dialyzed first against 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 1 M NaCl then extensively against 10 mM Tris-HCl (pH

7.5), 1 mM EDTA (TE). ^{3}H-labeled ØX DNA was prepared by adding [^{3}H] thymidine to 0.2 µCi/ml 10 minutes after infection. Additional purification of the DNA by alkaline sucrose sedimentation had no effect on the results.

Enzymes. The rat liver DNA nicking-closing enzyme (untwisting enzyme, type I topoisomerase) was purified by B. L. McConaughy as previously described (18). Electrophoresis of the purified enzyme in polyacrylamide gels containing SDS indicated that the enzyme was approximately 90% pure with a molecular weight of 70,000. The enzyme was stored in 70 mM potassium phosphate buffer (pH 7.4) (K^{+} concentration 0.12 M), 1 mM EDTA, 0.5 mM dithiothreitol (DTT), 10% glycerol at 0°. Untwisting of superhelical closed duplex DNA was measured by the DNA filter binding assay previously described (18). In reactions with duplex DNA the nicked form was quantitated by alkaline sucrose sedimentation as previously described (15). T4 polynucleotide kinase was purchased from New England Biolabs and stored at -20°. Proteinase K was purchased from Beckman and stored at 1 mg/ml in 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM $CaCl_2$ at 0°.

Enzyme reactions. Reaction mixtures for breakage of ssDNA by the nicking-closing enzyme contained, in addition to the stated amounts of enzyme and DNA, 5 mM Tris-HCl (pH 7.5), 35 mM potassium phosphate (pH 7.4) 1 mM EDTA, 0.25 mM DTT and 5% glycerol. Incubation time, temperature and method of stopping the reaction are described in the text. For end-labeling of broken strands with ^{32}P by polynucleotide kinase, reactions (50 µl) containing 5 µl of the products of the breakage reaction in 70 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.5 µM [γ ^{32}P] ATP (2500 Ci/mM) and 2 units of polynucleotide kinase were incubated for 3 hours at 0°. The kinase reactions were terminated by adding excess EDTA. Digestion with proteinase K (90 µg/ml) was carried out at 37° for 30 minutes.

Analysis of products. For alkaline sucrose sedimentation of ØX ssDNA, samples were diluted to 0.15 ml in 0.25 N NaOH and layered onto 5 to 20% sucrose gradients containing 0.25 N NaOH, 0.75 M NaCl and 5 mM EDTA. The gradients were centrifuged in the Beckman SW56 rotor at 55,000 rpm for 5 hours at 20°. Fractions were collected and radioactivity was determined as described (20). Alkaline CsCl equilibrium centrifugation analysis of protein-DNA complexes after end-labeling with ^{32}P by polynucleotide kinase was as previously described (12). Samples were prepared for agarose gel electrophoresis by adding one-fifth volume of 20% ficol, 0.25% bromophenol blue. Electrophoresis was carried out in a vertical 1.4% agarose gel (10 cm long, 14 cm wide, 0.38 cm thick) in Tris-borate-EDTA buffer (pH 8.3) (21) for

5 hours at 80 volts (unless otherwise indicated). Gels were stained in 0.5 µg/ml ethidium bromide for 20 minutes and photographed with UV illumination using Type 52 or 55 Polaroid film and an orange UV filter. To quantitate the amount of ^{3}H-labeled DNA in a band, the band was excised and placed in a glass scintillation vial with 1 ml of Soluene 350. After incubation overnight at 50° the vial was vortexed briefly, cooled to room temperature and 10 ml of toluene-Ominfluor (NEN) added. The vial was again vortexed and then counted in a Beckman LS-230 scintillation counter.

RESULTS

Breakage of single-stranded DNA by the nicking-closing enzyme.

The breakage of circular ØX ssDNA can be measured by alkaline sucrose sedimentation. Fig. 1a shows the sedimentation profile of circular ^{3}H-

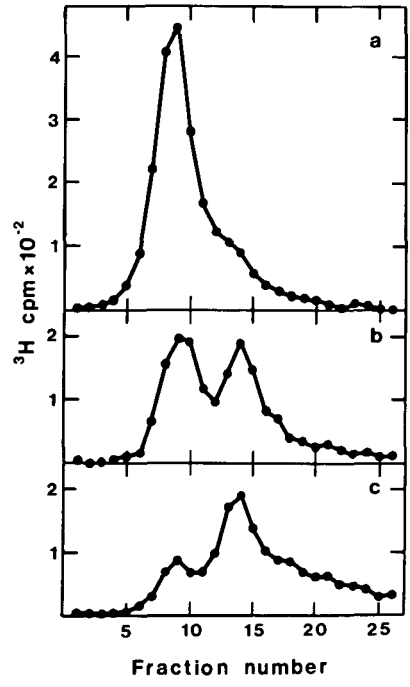

Figure 1. Alkaline sucrose sedimentation of ØX ssDNA broken by the nicking-closing enzyme. ^{3}H-labeled ØX ssDNA (0.2 µg, 7 X 10^{3} cpm/µg) was incubated at 24° with no enzyme (a) and with 0.15 µg of enzyme for 10 minutes (b) and 30 minutes (c). Reactions were stopped by adding SDS to 0.5% and digested with proteinase K prior to sedimentation. Sixty-two 3-drop fractions were collected from the bottom; only the first 26 fractions are shown.

labeled ØX ssDNA (18S) which contained 20% broken strands (16S). In 10 minutes at 24°, incubation of 0.2 µg of ØX ssDNA with 0.15 µg of nicking-closing enzyme resulted in breakage of approximately 50% of the circles (Fig. 1b). After a 30 minute incubation more of the circles were broken and slower sedimenting material which resulted from multiple breaks per molecule was evident (Fig. 1c).

Single-stranded circles and linears are also separated by agarose gel electrophoresis. Using the buffer system described in Materials and Methods, unit linears and smaller fragments migrate more rapidly than the circles (Fig. 2). The resolution of small discrete fragments generated in the reaction with nicking-closing enzyme suggests that multiple sites exist at which the enzyme preferentially breaks the DNA. Similar results were seen with ssDNA prepared from phage M13 virions (Fig. 2).

The agarose gel analysis provides a convenient means of quantitating the kinetics of the breakage reaction. Using ^{3}H-labeled DNA, the bands containing circles were excised from the gel and the amount of radioactivity determined. Fig. 3 shows the kinetics of breakage in 0.06 M K^{+} at 0°, 24° and 37° expressed in terms of the percent unbroken circles. There was no detectable breakage at 0°. Breakage occurred over a range of K^{+} concentrations from 0.01 to 0.15 M with 0.05 M being near the optimum at 37° (data not shown).

Single-stranded DNA broken by the nicking-closing enzyme contains a free 5'-hydroxyl and bound protein.

Studies on the nicked form isolated from reactions of the purified nicking-closing enzyme and duplex DNA have shown that the broken strand contains free 5'-hydroxyl and protein covalently attached to the 3' end (11,12). We investigated whether the breakage of ssDNA generates a complex with similar structure.

To test for the generation of a free 5'-hydroxyl in the breakage reaction, ^{3}H-labeled ØX ssDNA was reacted with nicking-closing enzyme and then placed on ice. The conditions were adjusted for labeling with ^{32}P by polynucleotide kinase and the incubation was continued for 3 hours at 0°. These conditions minimize the exchange reaction by the kinase (22) so that only free 5'-hydroxyl ends should be labeled with ^{32}P. After stopping the kinase reaction with EDTA the products were digested with proteinase K and then sedimented in alkaline sucrose. As seen in Fig. 4, single-stranded linears were labeled with ^{32}P indicating that the broken strands contained 5'-hydroxyl. If the initial 10 minute reaction with nicking-

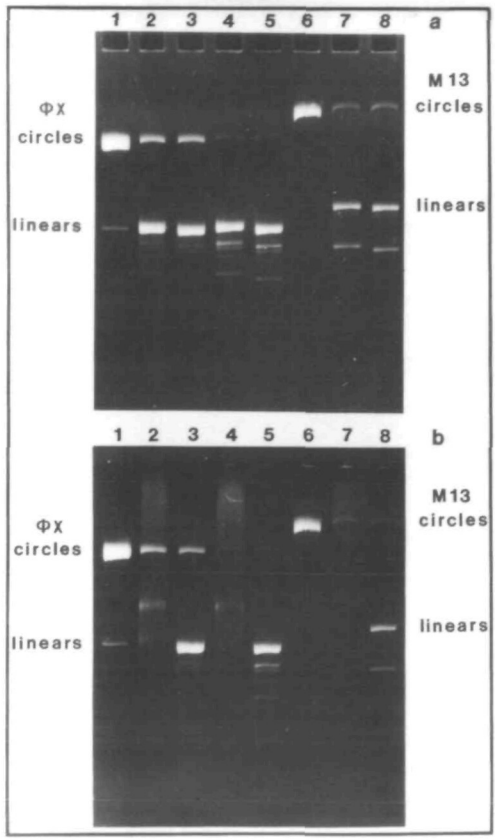

Figure 2. Separation of circular and linear forms of single-stranded DNA by agarose gel electrophoresis demonstrating breakage by the nicking-closing enzyme. Parallel identical samples were run in gels a and b, and the gels were the same but for the addition of 0.01% SDS to the gel and buffer in a. Lanes 1 and 6 show untreated ØX ssDNA and M13 ssDNA respectively. In lanes 2 through 5, ØX ssDNA (25 µg/ml) was reacted with nicking-closing enzyme (1.75 µg/ml lanes 2 and 3, 3.5 µg/ml lanes 4 and 5) under standard conditions for 30 minutes at 37° in a final volume of 50 µl. Lanes 7 and 8 contain a similar reaction with M13 ssDNA (40 µg/ml) and nicking/closing enzyme (3.5 µg/ml). Reactions were terminated by adding 5 µl of 5% SDS; 13 µl samples were electrophoresed in 1.6% agarose at 100 volts for 6 hours. Samples run in lanes 3, 5, and 8 were treated with proteinase K prior to electrophoresis.

closing enzyme was carried out at 0° (Fig. 4b) or nicking-closing enzyme omitted (data not shown) then neither breakage nor ^{32}P incorporation into preexisting broken strands was detected. The data suggests that the breakage by the nicking-closing enzyme occurs during the initial incubation,

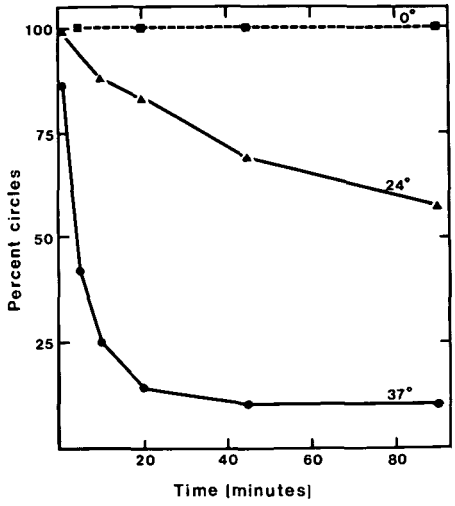

Figure 3. Kinetics of breakage of ØX ssDNA by the nicking-closing enzyme at various temperatures. Equal volumes of ^{3}H-labeled ØX ssDNA (25 µg/ml in TE) and nicking-closing enzyme (3.5 µg/ml in 70 mM phosphate buffer) were mixed and incubated at either 0°, 24° or 37°. At the indicated times 10 µl samples were removed and stopped by adding SDS to 0.5% followed by proteinase K digestion. After electrophoresis in 1.4% agarose the radioactivity in bands containing circular DNA was determined as described in Materials and Methods. Results are expressed as the percent of remaining circles.

ruling out the possibility that breakage only occurs during or after the proteinase K treatment.

The results shown in Fig. 2 suggested that the broken strands contain bound protein. When electrophoresis was carried out in the presence of SDS (Fig. 2a) the DNA fragments in samples which were treated with proteinase K migrated slightly faster than those in untreated parallel samples (compare lanes 2 and 3, 4 and 5, 7 and 8). This difference was most evident for smaller fragments. When SDS was omitted from the gel the non-deproteinized fragments failed to run as discrete bands (Fig. 2b).

In order to confirm the presence of protein bound to the broken strands the products of the reaction with ØX ssDNA were end-labeled with ^{32}P as described above and analyzed by equilibrium centrifugation in alkaline CsCl (Fig. 5). The ^{32}P-labeled broken strands banded at a lower density than the marker ^{3}H-labeled ØX DNA (Fig. 5a). Treatment of the products with proteinase K prior to centrifugation resulted in the co-banding of the

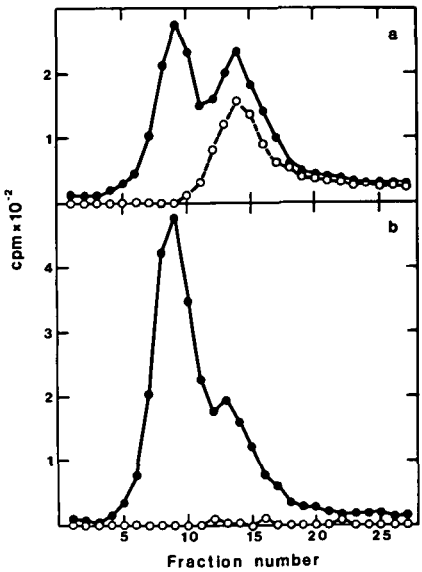

Figure 4. Alkaline sucrose sedimentation of ^{3}H-labeled ØX ssDNA end labeled by polynucleotide kinase with ^{32}P. ^{3}H-labeled ØX ssDNA (0.2 µg, 7 X 10^{3} cpm/µg) and nicking-closing enzyme (0.15 µg) were incubated for 10 minutes at either 24° (a) or 0° (b), both reactions were then placed on ice and the reaction conditions adjusted for labeling with ^{32}P as described in Materials and Methods. The kinase reaction was terminated by adding excess EDTA and the products digested with proteinase K prior to sedimentation. Only the bottom 27 of 62 fractions are shown. Sedimentation is from right to left. Open symbols, ^{32}P; closed symbols, ^{3}H.

^{32}P-labeled material and the marker (Fig. 5b), indicating that the lower buoyant density was due to bound protein. The observed shift in buoyant density is consistent with the predicted shift in buoyant density for one enzyme molecule bound per strand of DNA (11).

Breakage of ssDNA is stoichiometric and inactivates the enzyme.

If the breakage of ssDNA by the nicking-closing enzyme were accompanied by covalent attachment of the enzyme to the DNA then one might expect that the bound enzyme would be incapable of catalyzing additional ssDNA breakage, and therefore, the amount of breakage should be directly proportional to the amount of enzyme in the reaction. Likewise, subsequent activity with duplex DNA, after incubation of the enzyme with ssDNA, should be reduced.

The time course for breakage of ssDNA measured at three different

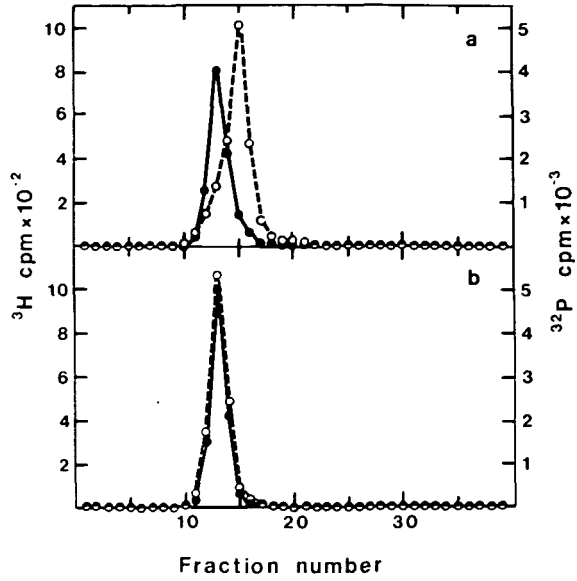

Figure 5. Alkaline CsCl equilibrium centrifugation of DNA protein complexes. Unlabeled ØX ssDNA (2.5 µg) and nicking-closing enzyme (0.15 µg) were incubated at 37° for 30 minutes under standard breakage conditions and then placed on ice. End-labeling with ^{32}P was as described above and the reaction was stopped by adding EDTA. The reaction was split and half was treated with proteinase K. The samples were then centrifuged to equilibrium in alkaline CsCl in the presence of ^{3}H-labeled ØX ssDNA as a marker. Fractions were collected onto glass fiber filters, washed and counted as previously described (12). a. no proteinase K treatment; b. digested with proteinase K prior to banding. Open symbols, ^{32}P; closed symbols, ^{3}H.

enzyme concentrations is shown in Fig. 6. ^{3}H-labeled ØX ssDNA was reacted with two-fold increasing amounts of nicking-closing enzyme. At the indicated times samples of the reactions were stopped with SDS. The products were digested with proteinase K and the fraction of unbroken circles quantitated by agarose gel electrophoresis as described above. The results are expressed both in terms of the percent of the circles remaining (Fig. 6a) and as the average number of nicks per molecule calculated using the Poisson distribution function (Fig. 6b). This average is probably an over-estimate at higher ratios of nicking-closing enzyme to DNA because the sites of breakage on the ØX ssDNA are not random (Fig. 2). Nevertheless, at the two higher enzyme concentrations the rate of breakage was greatly decreased after 20 minutes

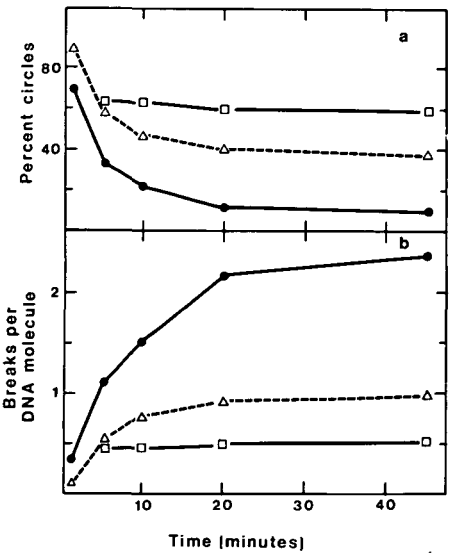

Figure 6. Time course of nicking reaction with increasing enzyme concentration. ^{3}H-labeled ØX ssDNA (12.5 µg/ml) was incubated with nicking-closing enzyme (0.43 µg/ml, squares; 0.85 µg/ml, triangles; 1.7 µg/ml, closed circles) at 37°. At the indicated times 10 µl samples were stopped by adding SDS to 0.5% and digested with proteinase K. After agarose gel electrophoresis bands containing circles were excised and radioactivity quantitated as described in Materials and Method. Results are expressed both as the percent of unbroken DNA (a) and as the average number of breaks per molecule of DNA (b) (see text).

and the number of breaks per molecule were approximately doubled as the enzyme concentration was doubled.

From the data given in Fig. 6 the fraction of the total enzyme molecules involved in the breakage of ssDNA can be estimated. At the three increasing enzyme concentrations (Fig. 6), incubation resulted in an average of 0.5, 0.9 and 2.2 breaks per molecule of DNA at 20 minutes. Assuming 90% purity of the nicking-closing enzyme and using approximate molecular weights of 7×10^4 for the enzyme and 1.8×10^6 for ØX ssDNA, the increasing concentrations of enzyme correspond to molecular ratios of enzyme to DNA of 0.8, 1.6, and 3.2, respectively. For the two reactions with an average of less than one break per molecule division of the number of breaks per DNA by the molecular ratio gives a value of 0.6 breaks per enzyme molecule. That is, approximately 60% of the enzyme molecules could have reacted with ssDNA.

Using duplex DNA as a substrate to assay residual enzyme activity, the decrease in activity, as a result of incubation with ssDNA, was measured. The activity with duplex DNA substrate can be measured either by estimating the percent of nicked form when the reaction is terminated with SDS (1%) (Fig. 7) or by assaying the relaxation of superhelical circular DNA (18). The decrease in duplex nicking activity after incubation with ØX ssDNA is shown in Fig. 7. Some loss of activity also occurred in the absence of ssDNA when the enzyme was incubated under the same conditions of salt concentration and temperature. Assuming a similar loss of activity occurred in the reaction with ssDNA as was observed in the control without DNA, we estimate that the nicking activity on duplex DNA was decreased by an additional 65% after a 15 minute reaction with ssDNA. This 65% decrease could be a minimal estimate since the enzyme is more stable in the presence of duplex DNA than in its absence (unpublished observations). A similar analysis of the residual relaxing activity after incubation of the enzyme with ØX ssDNA was carried out. In this case the ability of the enzyme to relax superhelical SV40 DNA,

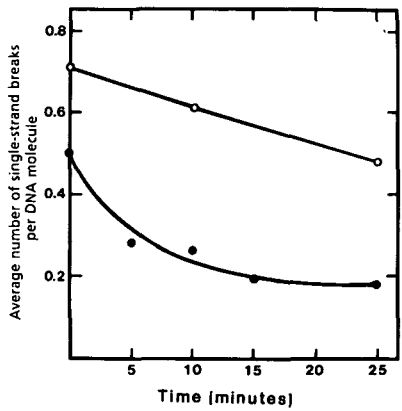

Figure 7. Decrease in duplex nicking activity by the nicking-closing enzyme during incubation of the enzyme with ssDNA. Nicking-closing enzyme (18 µg/ml) was incubated in the absence (open circles) or presence (closed circles) of ØX ssDNA (50 µg/ml) at 37°. At the indicated times 10 µl sample were removed and mixed with 10 µl of ^{3}H-labeled SV40 DNA (30 µg/ml) in the same buffer. After an additional 10 minutes at 24° the reactions were stopped by rapidly mixing with 20 µl of 2% SDS followed by proteinase K digestion. The conversion of form I to form II was detected and quantitated by alkaline sucrose sedimentation. The results are expressed as an average number of nicks per molecule of SV40 DNA as calculated from the fraction of unnicked SV40 DNA using the Poission distribution function. All numbers were corrected for preexisting form II in the SV40 DNA.

as measured by the DNA filter binding assay (18), was diminished by 60% in 20 minutes (data not shown).

DISCUSSION

Several observations suggest that the observed breakage of ssDNA is due to action of the nicking-closing enzyme and not a contaminating nuclease in the enzyme preparation. The broken strands contain covalently bound protein which is an unlikely product of a nuclease. The breakage of single-strands appears to be proportional to the amount of added enzyme and addition of Mg^{++} to 5 mM or 10 mM does not increase breakage (data not shown). Finally, the increase in the breakage of ssDNA is paralleled by a decrease in the activity of the nicking-closing enzyme on duplex DNA. Taken together the evidence strongly suggests that the activity which breaks ssDNA is the same as the one responsible for the previously measured activities on duplex DNA. In support of this conclusion, reclosure of the broken strands under certain conditions of salt and temperature has been observed (Been and Champoux, manuscript in preparation).

Although the rat liver nicking-closing enzyme can both nick double-stranded and break ssDNA to yield a DNA-protein complex, the two reactions are different in at least three respects. (i) The level of broken ssDNA increases with time whereas the amount of nicked complex detected in reactions with duplex DNA is constant once the reaction has come to thermal equilibrium (15). (ii) The breakage of ssDNA described here does not require denaturing conditions (pH extremes or detergents) which have previously been found necessary to detect DNA-protein complexes in reactions with duplex DNA (6,13,14,15). In the reaction with ØX ssDNA the 5'-hydroxyls are available to phosphorylation by polynucleotide kinase before the reaction is stopped with a denaturing treatment. This result demonstrates that the observed breakage of ØX ssDNA is not a consequence of the denaturing treatment, but rather is probably the result of a reaction which aborted before reclosure could occur (see below). Thus strand breakage by a DNA topoisomerase under nondenaturing conditions has been demonstrated. (iii) For a given quantity of nicking-closing enzyme in a reaction, the amount of broken ssDNA is much higher than the amount of nicked DNA detected when a reaction with duplex DNA is stopped under conditions that yield the nicked form. With ssDNA the percent of enzyme molecules which form broken DNA-protein complexes has been estimated from the data in Fig. 5 to be approximately 60%. From the stoichiometry of nicking of duplex DNA (Fig. 7), one calculates that only about 4%

of the enzyme molecules have nicked the DNA. There are two possible explanations for the lower nicking value observed with duplex DNA. The level of nicking may represent the actual amount of nicked intermediate in the overall nicking-closing reaction. If this explanation is true and given that the enzyme is tightly bound to the DNA under these low salt conditions (23), then the equilibrium in the nicking-closing reaction favors the closed form. Alternatively, the nicking of duplex DNA may be induced by the denaturing conditions used to stop the reaction and the low level may simply reflect the efficiency of this process.

Our current view of the breakage reaction with ssDNA is that it is the result of an aborted reaction where the enzyme is unable to complete the nicking-closing cycle due to a loss of positioning of the two termini for reclosure. Since the enzyme only breaks ssDNA at a limited number of specific sites (Fig. 2 and unpublished results) secondary structure in the molecule could be important for this aborted reaction. Possibly a limited amount of secondary structure is required for recognition and binding of the enzyme to the DNA and breakage only occurs at sites where the extent of secondary structure is not sufficient to allow for reclosure. This hypothesis would predict that some regions of rather extensive secondary structure in ssDNA could be a substrate for cycles of nicking and closing rather than breakage. The sequestering of enzyme at these regions may explain why there is always some residual activity after the breakage reaction with ssDNA (see Fig. 7).

The similarity between the ØX174 cistron A gene product (cisA protein) and DNA topoisomerases has been noted previously (3); both the cisA protein and the topoismerases appear to act by a mechanism involving a covalent protein-DNA intermediate (24). In addition both the cisA (24) and the related A* protein have been shown to break ØX ssDNA (25) and it has been reported that single-strands of ØX DNA broken by the cisA protein can be recircularized (24). Whether the in vitro breakage of ssDNA represents more than a superficial similarity of these enzymes remains to be determined.

ACKNOWLEDGEMENTS

We would like to thank B. McConaughy and B. Brewer for their critical reading of the manuscript. This work was supported by National Institute of Health Research Grant GM23224 and in part by USPHS 1-T32-GM-07270.

Note: After this work was submitted we became aware of a recent paper by Prell and Vosberg (Eur. J. Biochem. (1989) 108, 389-398) in which they demonstrated that calf thymus nicking-closing enzyme breaks fd ssDNA and that the broken strands bind to glass fiber filters as a result of covalently bound protein. Most importantly, they showed that the breakage of ssDNA and formation of the protein-DNA complex is not the result of the alkaline treatment used in their assay.

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