Evidence for an intermediate with a single-strand break in the reaction catalyzed by the DNA untwisting enzyme
(superhelical simian virus 40 DNA/DNA relaxation/nicking-closing enzyme/DNA replication)

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Communicated by A. H. Doermann, July 28, 1976

ABSTRACT The DNA untwisting enzyme relaxes covalently closed circular DNAs by the sequential breaking (nicking) and closure of one strand of the duplex. The use of highly purified enzyme from rat liver nuclei at very high protein concentrations has permitted the detection of the nicked intermediate in the reaction. The nicking of closed circular simian virus 40 DNA was measured by alkaline sucrose gradient sedimentation or by equilibrium centrifugation in CsCl gradients containing propidium diiodide. The following observations support the hypothesis that the nicked DNA represents an intermediate in the untwisting reaction. The extent of nicking does not increase with time. Nicking is observed in the range of salt concentrations where the enzyme is active (0.01-0.35 M KCl), but is not observed at 0.50 M KCl, where enzyme activity is undetectable. The nicked DNA that is generated during the reaction carried out in low salt rapidly disappears if the KCl concentration is raised to 0.50 M. At constant enzyme concentration, the number of nicks in the reaction mixture is independent of DNA concentration in the range from 3 to 14 µg/ml. The addition of an excess of unlabeled DNA to a reaction initially containing labeled nicked DNA partially chases the label from the nicked intermediate into covalently closed circular DNA.

The DNA untwisting enzyme from cultured mouse cells or rat liver cells removes both negative and positive superhelical turns from a covalently closed DNA substrate (1, 2). A similar enzyme activity has now been identified in a wide variety of eukaryotic cell types and referred to as an ω protein (3, 4), a DNA relaxing enzyme (5, 6), and a nicking-closing enzyme (7, 8). As suggested by the latter name, these enzymes must act to introduce a transient nick (break) in one strand of duplex DNA in order to effect the relaxation process. The reaction sequence can be formulated as follows:

\[ E + DNA_{\text{closed}} \rightarrow E(DNA_{\text{nicked}}) \rightarrow E(DNA_{\text{closed}}) \]

Free enzyme (E) binds to DNA to form an enzyme-substrate complex which is then reversibly nicked. During the lifetime of the nicked intermediate, free rotation of one strand of the helix relative to the other strand is possible (9). The driving force for the relaxation process derives from the free energy associated with the superhelical turns in the closed molecule (10).

Heretofore, the presence of a nicked intermediate has not been demonstrated and its existence has only been inferred from the nature of the overall reaction. In this paper, evidence for a nicked intermediate in the untwisting reaction is presented.

MATERIALS AND METHODS

General. The sources for all the reagents and materials have been given (1, 11). The procedure for the preparation of 3H-labeled DNA of simian virus 40 (SV40) was the same as previously described (11) except the cells were labeled at 10 µCi/ml with [3H]thymidine (30 Ci/mmol). The specific activity of the DNA was 4 × 10^6 cpm/µg. The procedures for the preparation of singly-nicked SV40 DNA by treatment with pancreatic DNase and for equilibrium centrifugation in CsCl-propidium diiodide (PrpI2) gradients have been described (11).

DNA Untwisting Enzyme. The procedure for the purification of the enzyme from rat liver nuclei is presented elsewhere (12). Electrophoresis of the purified enzyme in polycrylamide gels containing sodium dodecyl sulfate indicated that the enzyme was approximately 90% pure and has a molecular weight of 68,000. The enzyme fraction used in this study contained 44 µg/ml of protein in 70 mM potassium phosphate buffer (pH 7.4) (K+ concentration 0.12 M), 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol. Enzyme activity was measured by the DNA filter binding assay, which is described in detail elsewhere (12).

Enzyme Reactions. Each reaction mixture contained, in the stated volume, 10 µl of the highly purified DNA untwisting enzyme. The concentration of SV40 DNA and the final K+ concentration (determined entirely or in part by the enzyme buffer) is given for each experiment. Those reactions carried out in a volume of 100 µl contained, in addition to the other components, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA. The mixtures were incubated for the indicated periods of time in small plastic tubes at 37°C. The reactions were stopped by dilution to 0.15 ml with a concentrated mixture of NaOH and EDTA to give final concentrations of 0.12 M NaOH and 10 mM EDTA. In one experiment (Fig. 3a) the reaction was stopped by dilution of the sample into 0.15 M Na3PO4, 10 mM EDTA (uncorrected pH 12.4). Under these conditions control experiments showed that nicked molecules were completely denatured, whereas covalently closed circles were only partially denatured and thus regained their native duplex structure on neutralization (13).

Alkaline Sucrose Sedimentation. The samples were layered onto 5-20% sucrose gradients containing 0.25 M NaOH, 0.75 M NaCl, 1 mM EDTA. The gradients were centrifuged in the Beckman SW56 rotor at 55,000 rpm for 80 min at 18°C. Fractions were collected and the radioactivity was determined as described (14).

RESULTS AND DISCUSSION

Salt dependence of DNA untwisting enzyme

The DNA untwisting enzyme was originally found to be active in the absence of added divalent cations, but to require monovalent cations (either Na+ or K+) at a concentration of approximately 0.20 M (1). Fig. 1 shows a more detailed analysis of the salt requirement of the enzyme. In agreement with our previous results and the results of others (4, 6, 8), the enzyme is maximally active between 0.15 M and 0.20 M KCl. At lower
The reaction during preparation.

Enzyme reaction intermediate alkali, (Exp. DNA substrate) Kaline sucrose the mM nicked circles, enzyme. Untwisting fact serves as either as a closed circular valently consequence at 0.25 M.

Nicked intermediate

Under alkaline denaturation conditions the two strands of covalently closed circular SV40 DNA remain interwound, and as a consequence sediment approximately three times as fast as either single-stranded circular or linear molecules (15, 16). This fact serves as the basis of the assay for nicking by the DNA untwisting enzyme.

A sample of 3H-labeled SV40 DNA, initially containing 19% nicked circles, was treated for 5 min at 37° in the presence of 60 mM K+ with purified DNA untwisting enzyme (final protein concentration was 22 μg/ml) (Exp. 2a, Table 1). Fig. 2a shows the distribution of labeled DNA after centrifugation in an alkaline sucrose gradient. Under these conditions 85% of the substrate DNA was nicked at the time the reaction was stopped with alkali, whereas no increase in the proportion of nicked DNA was observed in a parallel reaction carried out in 0.50 M K+ (Exp. 2b, Table 1). In the reaction carried out in low salt, the proportion of nicked DNA does not increase if the time of incubation is extended to 30 min (data not shown). This suggests that the observed nicking may be due to a constant level of nicked intermediate present during the course of the untwisting reaction and not due to an endonuclease contaminant in the enzyme preparation.

In order to rule out the possibility of an endonuclease contaminant, it is necessary to demonstrate that the nicks generated during the course of the reaction can be resealed. If the nicking does reflect the steady-state level of a nicked intermediate and if the enzyme does not bind to DNA in the presence of high salt (KCl > 0.30 M), then the addition of high salt to a reaction containing the nicked DNA would be expected to shift the equilibrium in favor of free enzyme and closed DNA (see reaction sequence above). To test this possibility, I carried out a two-step reaction. Enzyme and DNA were first incubated for 5 min under conditions identical to those for the reaction analyzed in Fig. 2a. At 5 min the reaction mixture was diluted 5-

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* Each reaction mixture contained, in the specified volume, the indicated concentrations of K⁺ and native SV40 DNA, and 10 μl of purified DNA untwisting enzyme (see Materials and Methods). The mixtures were incubated under the initial conditions for 5 min at 37°C and, where indicated, shifted to the final conditions and incubated at 37°C for another 5 min. The reactions were stopped and samples analyzed for the percent of nicked DNA by alkaline sucrose gradient centrifugation (see Materials and Methods). In Exp. 4c the DNA concentration was increased by the addition of unlabeled SV40 DNA. Therefore the percent nicked refers only to the labeled component present throughout the incubation.

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![Fig. 1](image-url) Salt dependence of the DNA untwisting enzyme. The activity of the purified enzyme was determined using the filter assay (2). The reactions contained 20 mM Tris-HCl (pH 7.5) and 1 mM EDTA in addition to the indicated concentrations of KCl. No activity was detectable for the symbols given in parentheses. The values plotted indicate the lower limit of detection for the assay.
fold into a buffer containing 0.50 M KCl and the incubation continued for another 5 min (Exp. 2c, Table 1, and Fig. 2b). The proportion of nicked DNA returned to essentially the value (22%) found for the untreated DNA. Control experiments given in Table 1 (2d and 2e) show that this effect is primarily due to the addition of the salt and is not simply a consequence of the dilution of the reaction.

These results indicate that the DNA unwinding enzyme itself and not a contaminant endonuclease is responsible for the observed nicking. Although no enzyme activity, as measured by the DNA unwinding assay or by the nicking assay, is demonstrable in the presence of high salt, the resealing step in the reaction can occur. Apparently, once the enzyme resauses the nick, the high salt causes it to dissociate from the DNA and remain dissociated. The fact that virtually all of the nicks are resealed indicates that the high salt does not dissociate the enzyme from the nicked form of the DNA. This suggests that there may be a stronger association of the enzyme with the nicked form than with the closed form of the DNA (see below).

The effect of salt concentration on the amount of nicked intermediate present during the enzyme reaction has been examined. The nicked intermediate was detected at all KCl concentrations where enzyme activity is detectable. However, a greater proportion of the substrate DNA was nicked at K+ concentrations ranging from 0.01 M to 0.10 M than in the range from 0.15 to 0.20 M, where the enzyme is most active. This may result from an effect of ionic strength on the equilibrium distribution of nicked and closed substrate DNA. This phenomenon is being investigated further.

**Analysis by CsCl-PrpI2 centrifugation**

By banding the reaction products in a CsCl-PrpI2 gradient, one can determine the superhelix density (17) of the closed circular DNA in addition to measuring the extent of nicking. A reaction mixture similar to the one described in Exp. 2a in Table 1 was incubated at 37°C for 5 min. In one aliquot of the mixture, the reaction was stopped by an alkaline treatment which completely denatured any nicked molecules while only partially denaturing closed circles. With this treatment, closed circular DNA returns to its native duplex structure after neutralization (see Materials and Methods). After centrifugation to equilibrium in CsCl containing PrpI2 (Fig. 3a), the single-stranded DNA formed a band at the expected position (18) intermediate between the open and closed forms of the 14C-labeled marker SV40 DNA. From the amount of single-stranded DNA in the gradient, the substrate was calculated to be 78% nicked at the time the reaction was stopped, thus confirming the results obtained by alkaline sucrose gradient sedimentation.

It is noteworthy that the substrate DNA, which was closed at the time the reaction was stopped, had an increased buoyant density in CsCl-PrpI2 (Fig. 3a), indicating that it was-
pletely relaxed (16). This result rules out the possibility that the enzyme is nicking but not resealing the substrate under these conditions since, if this were the case, the unnicked DNA would still be superhelical.

In a second aliquot of the mixture, the reaction analyzed in Fig. 3a was stopped by dilution directly into 4.5 M CsCl, without any further incubation. From the distribution of labeled DNA in the resulting CsCl-PrpI2 gradient (Fig. 3b) it can be seen that, even in the presence of 4.5 M CsCl, the nicked DNA was resealed. As expected, all of the closed circular DNA was completely relaxed.

Dependence of nicking on DNA concentration

A series of enzyme reactions were carried out with increasing DNA concentrations. As can be seen from Exp. 3, Table 1, the proportion of nicked molecules present in the reaction mixture decreases as the DNA concentration is increased. Assuming that nicking is a random process, one can calculate, with the use of the Poisson distribution, the average number of nick per DNA molecule in each reaction mixture. Multiplying this number by the DNA concentration yields the concentration of nicks in each reaction. After correcting for the preexisting nicks in the substrate DNA, it was found that the concentration of enzyme-induced nicks was independent of the DNA concentration over the range tested. This result suggests that the lowest DNA concentration used (3.4 μg/ml) represents a saturating level of substrate. The concentration of nicks in the reaction mixture corresponded to approximately one nick for every 100 molecules of enzyme present. From the loss of activity during purification and storage, we estimate that between 1% and 10% of the enzyme molecules in this preparation are active (unpublished experiments). Therefore, the reaction mixture contained between 1 and 10 active enzyme molecules per nick.

From the effects of DNA concentration described above, one can calculate that at a DNA concentration of 180 μg/ml, the amount of nicked DNA in the reaction should only increase from the background level of 19% to a value of 24%. The observed increase (Exp. 4a, Table 1) is very close to the predicted value. This result suggested that the addition of a large excess of unlabeled DNA to the usual reaction mixture after 5 min of incubation might "chase" the labeled DNA out of the nicked intermediate. Exp. 4, Table 1 shows the results of such an experiment. At the end of 5 min, 79% of the labeled DNA (at 6.8 μg/ml) was found to be nicked (Exp. 4b, Table 1). At this time, the total DNA concentration was increased to 180 μg/ml by the addition of unlabeled DNA and the incubation continued for another 5 min. At the end of the incubation the amount of labeled DNA that was nicked had decreased to 44% (Exp. 4c, Table 1). This result indicates that at least some of the labeled DNA can be chased out of the nicked intermediate into closed circular DNA by the addition of excess unlabeled DNA. The failure to observe a decrease to the same level as was found in the reaction carried out initially in the presence of the high DNA concentration might be due to a slow rate for the closure step of the reaction. Alternatively, this result may be due to such a tight association of the enzyme with the substrate DNA that the same molecules are repeatedly nicked and sealed before the enzyme dissociates and becomes free to bind to other DNA molecules in the solution.

Nicked DNA as a substrate for the enzyme

We have previously shown that the DNA untwisting enzyme will not act as a ligase if provided with a substrate DNA containing a single-stranded interruption bounded by a 5'-phosphate and a 3'-hydroxyl (1). This experiment was repeated under the conditions of high enzyme concentration used here and the same result was obtained, even after incubations for up to 30 min at 37°. Although these data do not allow us to rigorously rule out other alternatives, we consider it likely that the enzyme is only capable of resealing the same nick that it generates. This hypothesis is consistent with the earlier suggestion that the enzyme might be covalently attached to one of the ends at the site of the nick, and in this way it would conserve the energy required for the closure step (1, 19).

I thank Patricia Bedinger for technical assistance and S. Falkow and B. McConaughy for helpful suggestions during the preparation of the manuscript. This work was supported by National Institutes of Health Research Grant GM23224 and by a grant from the University of Washington Graduate School Research Fund.