Action of ATP-dependent DNase from *Hemophilus influenzae* on Cross-linked DNA Molecules*

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The ATP-dependent DNase from *Hemophilus influenzae* digests double-stranded linear DNA molecules exonucleolytically while hydrolyzing large amounts of ATP to ADP. Various cross-linked linear duplex DNA molecules are partially resistant to the exonuclease action. Vaccinia DNA, containing natural terminal cross-links (probably in the form of terminal single-stranded loops), is much more slowly degraded than comparable "open-ended" DNA molecules, and ATP is consumed at a proportionately lower rate. It is postulated that the vaccinia DNA molecules undergo slow terminal cleavage by the single strand specific endonuclease activity of the enzyme, and are then rapidly degraded by the double strand exonuclease activity. Phage T7 DNA, containing an average of 100 4,5',8-trimethylpsoralen cross-links/molecule at random internal sites, is digested only to the extent of 2 to 3%.

However, ATP hydrolysis continues at a linear rate long after DNA digestion has ceased. A stable enzyme-DNA complex is formed as demonstrated by cosedimentation of DNA and ATPase activity in sucrose gradients. The hypothesis is advanced that the enzyme digests exonucleolytically to the first cross-link at each end of the DNA molecules where further movement is prevented. The enzyme then remains bound at the cross-links and functions continuously as an ATPase.

The general properties of the ATP-dependent DNase (exonuclease V) from *Hemophilus influenzae* have been described in previous papers from this laboratory (1-4) and its action on a variety of DNA substrates has been studied (3). Duplex linear DNA is rapidly degraded under standard reaction conditions, but duplex circular DNA is not attacked. The enzyme's classification as an exonuclease is based on this specificity. However, paradoxically it acts as an endonuclease activity on single-stranded DNA. Single-stranded circular DNA molecules are slowly cleaved even by highly purified preparations of enzyme (3, 5). This endonucleolytic activity is inhibited by the presence of linear duplex or single-stranded DNA both of which are preferred as substrates to the circular single-stranded form (3). Linear single-stranded DNA is digested at one-tenth the rate of duplex DNA molecules. DNA substrates in the order of enzyme preference are therefore duplex linear DNA > single-stranded linear DNA > single-stranded circular DNA > duplex circular DNA.

*Hemophilus* exonuclease V has two other enzymatic activities on duplex DNA in addition to nuclease activity: (a) it is a very active DNA-dependent ATPase and under standard reaction conditions as many as 30 to 40 ATP molecules are hydrolyzed to ADP per phosphodiester bond cleaved (2); (b) it possesses a DNA unwinding activity that converts double-stranded DNA into single-stranded DNA early in a reaction, prior to the accumulation of significant acid-soluble oligonucleotide products (4, 6). Putting these observations together, we have formulated a simple working model for the enzyme mechanism. We propose that enzyme initiates attack at the ends of duplex DNA, and then utilizes energy from ATP hydrolysis to move along the DNA and unwind regions of the molecule, releasing large partially or totally single-stranded fragments as cleavage occurs.

In this paper and two subsequent articles we describe evidence supporting the general features of this model. Here we analyze the action of exonuclease V on two kinds of DNA containing covalent cross-links that prevent effective strand separation: vaccinia virus DNA containing naturally occurring terminal cross-links (7), and DNA artificially cross-linked at random internal sites by 4,5,8-trimethylpsoralen (8). According to our hypothesis we would expect DNA cross-links to impede processive enzyme movement, thus decreasing DNA digestion and strand separation. We shall show: (a) that vaccinia DNA is resistant to digestion, and (b) that psoralen-cross-linked DNA is also resistant but binds to the enzyme and leads to prolonged hydrolysis of ATP. This uncoupling of the ATPase and DNase reactions with psoralen-cross-linked DNA...
is similar to that observed by Karu and Linn (9) using exonuclease V of Escherichia coli.

EXPERIMENTAL PROCEDURE

DNA Preparations—Unlabeled and [3H]labeled phage T7 DNAs were prepared as previously described (1). Vaccinia virus, simian virus (SV) 40 DNA, and adeno-associated virus were gifts of Paul Geshelin, Gary Berns, and Kenneth Berns, respectively.

Chemicals—[8-3H]ATP, 32.2 Ci/mmol, was obtained from New England Nuclear. 4,5,8-Triethylpsoralen was obtained from the Paul B. Elder Co., Bryan, Ohio. Agarose and ethidium bromide were obtained from Sigma Chemical Co.

Enzyme—Hemophilus ATP-dependent DNase (Fraction VI), 1780 units/ml, approximately 10,000 units/mg, was prepared as previously described (1), except for minor modifications to be described elsewhere. One unit of enzyme produces 1 nmol of acid-soluble oligonucleotides in 20 min in a standard reaction mixture. DNA-enzyme binding studies (10) show that 0.43 unit of enzyme will saturate the ends of 1 nmol of phage T7 DNA.

Preparation of 4,5,8-Triethylpsoralen Cross-linked DNA—Phage T7 DNA was cross-linked by treatment with 4,5,8-trimethylpsoralen essentially as described by Cole (8). The psoralen stock solution contained 0.2 mg/ml of psoralen in 95% ethanol. Reacton mixtures (0.25 ml) containing 20 m mol potassium phosphate buffer (pH 7.0), 30 mM NaCl, 25 nmol of T7 DNA, and various amounts of psoralen were irradiated for 1 h at 25°C with long wave (360 nm) ultraviolet light provided by a B100-A Blak-Ray lamp (Ultra-Violet Products, Inc., San Gabriel, Calif.) positioned at a 1.0-m distance. An additional filter (8 mm thick Corning Glass No. 5840) was interposed about 10 cm in front of the DNA sample. Under these conditions DNA in the absence of psoralen was not detectably cross-linked.

The mean number of psoralen cross-links introduced into the DNA was determined by measuring the amount of DNA that remained detectable after heat treatment and fast cooling. A cross-linked DNA sample was heated at 100°C for 1 min and immediately placed on ice. A 10-μ1 aliquot was analyzed by electrophoresis on a 0.6% agarose gel at 16 V/cm for 1 h. Under these conditions single-stranded DNA is clearly resolved from duplex DNA. The gel was stained with ethidium bromide and photographed, and the film negative was traced using a Joyce-Loebl Densitometer. Areas under the single- and double-stranded peaks were measured, applying a correction factor of 2.5 for the decreased ethidium bromide staining of single- versus double-stranded DNA. Assuming a Poisson distribution of cross-links, the number of cross-links/molecule was calculated from the single-stranded fraction \( \rho_s = e^{-\lambda} \). Under the conditions used, one cross-link was introduced per T7 molecule at a psoralen (P) to DNA (D) molar ratio of 80, and the relationship \( m = (1/80) (P/D) \) was shown to be valid at least P/D = 240. Generally, in the experiments reported, P/D was approximately 8000. If extrapolation is valid this corresponds to approximately 100 cross-links/molecule. We refer to this DNA as "heavily cross-linked DNA."

DNase and ATPase Assays—Assays were carried out in standard reaction mixtures (1) except as otherwise noted. Acid-soluble and acid-precipitable DNA radioactivities were determined by chromatography in 1 M HCl on polyethyleneimine-cellulose thin layer sheets as previously described (1). Conversion of ATP to ADP was measured by polyethyleneimine-cellulose chromatography in 0.5 M potassium phosphate buffer, pH 3.5, as previously described (2).

Agarose Gel Electrophoresis—DNA samples were analyzed on 0.8% agarose gels prepared in glass tubes (0.6 cm inside diameter by 12 cm length). The electrode buffer was 0.2 M glycerine, 0.015 M NaOH, pH 8.6. DNA samples (20 to 200 ng of DNA in a volume of approximately 10 μ1) contained 10% glycerol and a small quantity of bromphenol blue tracking dye. After electrophoresis at 8 to 10 V/cm for 1 to 2 h at room temperature, gels were removed, stained for 30 min in 0.5 μg/ml of ethidium bromide, 50 mM Tris/HCl, pH 8, and photographed under short wavelength ultraviolet light using Polaroid P/N type 55 film.

Sucrose Density Gradient Centrifugation—Velocity sedimentation of DNA or DNA-enzyme complexes was carried out on 5 ml of linear 5 to 20% sucrose gradients containing 0.1 M Tris/HCl, pH 8. Centrifugation was for 2 h at 48,000 rpm at 4°C in a Spinco SW 50 rotor. Fractions were collected and assayed as detailed under "Results."

RESULTS

Hemophilus Exonuclease V Activity on Vaccinia DNA—ATP-dependent DNase is classified as an exonuclease on double-stranded DNA but it degrades linear molecules but is inactive on circular forms. Recently Geshelin and Berns (7) discovered that the DNA extracted from vaccinia virus is a linear, duplex molecule containing terminal cross-links. The chemical structure of these cross-links has not been determined, but it appears likely that the ends are simply closed by short single-stranded loops of DNA (7). To determine whether exo V activity would be affected by such terminal cross-links, a reaction mixture containing vaccinia DNA, phage T7 DNA, adeno-associated virus DNA, SV40 form I DNA (supercoiled), and SV40 form II DNA (nicked circular) was incubated for 5 min at 37°C with enzyme and analyzed by agarose gel electrophoresis (Fig. 1). In the control sample without enzyme the five different DNA species were separated on the gel into five bands. In the sample treated with enzyme, the two bands corresponding to the linear duplex adeno-associated virus and T7 DNAs were no longer visible on the gels, while the other three bands appeared unchanged. Identical results were obtained in similar experiments in which each DNA was

![Fig. 1. Agarose gel electrophoresis of various viral DNA molecules treated with Hemophilus exonuclease V. The reaction mixture (30 μl) contained 20 ng each of adeno-associated virus (AAV), vaccinia, and T7 DNAs, 45 ng of a mixture of form I and II SV40 DNA, 100 mM Tris/HCl (pH 8), 10 mM MgCl₂, and 1 mM ATP. A 10-μl sample was removed at 0 min and added to 2 μl of 66% glycerol, 0.16 M EDTA. Then 4.4 units of exo V was added to the remaining reaction mixture. The reaction proceeded at 37°C for 5 min and was terminated by addition of 4 μl of glycerol/EDTA. Ten-microliter samples from the 0- and 5-min time points were electrophoresed on 0.6% agarose gels at 8 V/cm for 70 min at 23°C, then stained with ethidium bromide and photographed.](http://www.jbc.org/Downloaded from)
linked DNA was used. Incubation was at 37°C. Five-microliter samples
were taken at intervals and analyzed chromatographically for ATP
hydrolysis and acid-soluble 32P radioactivity as described under
"Experimental Procedures" and then used as a substrate for exo V in a reaction containing [3H]ATP (Fig. 2). Only about 2% of the cross-linked DNA was converted to
product (Fig. 2, Curve A) as compared to 60% conversion of non-cross-linked DNA to acid-soluble products (representing almost a limit product) in an otherwise identical control reaction (Fig. 2, Curve B). ATP hydrolysis, on the other hand, proceeded at a linear rate throughout the observed time period in the cross-linked DNA reaction (Fig. 2, Curve C), while in the control reaction, ATP hydrolysis reached a plateau within 10 min (Fig. 2, Curve D), roughly paralleling the kinetics of DNA digestion. Thus psoralen-cross-linked DNA is resistant to degradation but supports prolonged ATPase activity. This apparent uncoupling of ATPase from DNase activity on psoralen-cross-linked DNA has also been observed by Karu and Linn (9) using the exonuclease V from Escherichia coli. A reasonable interpretation of our results is that exo V degrades DNA up to the outermost psoralen cross-link, where it remains tightly bound, forming a DNA-enzyme complex having ATPase activity. We give evidence in support of this below.

**Enzyme Binding to Cross-linked DNA**—To test for formation of a stable complex between enzyme and psoralen-cross-linked DNA, a less than saturating amount (10) of *Hemophilus exo V* was preincubated for 3 min at 37°C with unlabeled, native T7 DNA or cross-linked T7 DNA in two separate complete reaction mixtures. Native T7 [32P]DNA was then added to each tube, and its degradation measured as an assay for any unbound, active exo V. Preincubation with native T7 DNA did not affect the activity of the enzyme, as shown by the rapid degradation of the added [32P]DNA (Fig. 3). However, preincubation with cross-linked DNA greatly reduced the available exonucleolytic activity, as shown by the slow degradation of the added [32P]DNA. This result is compatible with our proposal that the enzyme binds strongly to cross-linked DNA. An alternate explanation is that after contact with cross-linked DNA, enzyme dissociates into a form that cannot digest DNA but can act as an ATPase.

Actual binding was demonstrated by showing that enzyme activity cosediments with cross-linked T7 DNA in sucrose gradients. Heavily cross-linked T7 [32P]DNA (6 nmol) was incubated with an excess of enzyme (8.8 units) in a reaction mixture containing both ATP and Mg2+. The mixture was then layered onto a sucrose gradient and centrifuged to separate DNA from free enzyme. Fractions were collected and assayed for DNA, for ATPase activity and for DNase activity. The results are shown in Fig. 4. Most (85%) of the DNA was detected as a peak of 32P radioactivity centering in Fractions 15 to 17. This corresponds approximately to the position of intact T7 DNA, as determined by sedimentation on parallel gradients of similar reaction mixtures lacking either ATP or Mg2+, and indicates that there was little degradation of the cross-linked DNA. The small percentage (15%) of radioactivity near the top of the gradient may represent material produced by enzymatic degradation of the region between a DNA terminus and the outermost cross-link.

ATPase activity sedimented almost exactly with the nearly intact [32P]DNA at Fractions 14 through 18, except for a small shoulder extending upward in the gradient. DNase activity was not detected in the region of the intact DNA, but was found higher in the gradient at Fraction 25. Assuming that the [32P]DNA peak represents approximately full length molecules
vaccinia DNA is plotted in Curve D of Fig. 5. In 30 min about 40% of the vaccinia molecules were degraded so that no intermediate products were visible on the gels. However, the remaining 60% moved in the normal position of intact molecules. Slowing of the reaction probably reflects increasing competition of the oligonucleotide products for the available enzyme rather than a resistant fraction of vaccinia DNA. The structure of the terminal vaccinia cross-links is unknown; but we conclude that they are somewhat susceptible to attack by exo V, accounting for the low ATPase activity observed. It should be noted that under the conditions of our experiment, native T7 DNA is completely digested and the psoralen-crosslinked DNA is almost completely resistant (data not shown).

INTERPRETATION AND DISCUSSION

Cross-linked DNA molecules prove to be interesting substrates for exonuclease V. Terminally cross-linked vaccinia DNA is only slowly attacked and supports a correspondingly low rate of ATP hydrolysis (Figs. 1 and 5). Interpretation of these results is complicated by incomplete knowledge of the vaccinia cross link structure. However, closure of each DNA end by a short single-stranded loop is compatible with the known facts. The cross-links have been located within six nucleotides of the ends;4 furthermore, they can be cleaved by a single strand specific endonuclease contained in the vaccinia virion (7).

Assuming that the ends of vaccinia DNA are sealed by short loops, a simple mechanism based on established properties of Hemophilus exo V can be put forward to explain our observations (Fig. 6A). We propose that exo V cannot normally initiate at the ends of vaccinia DNA; therefore, no digestion or ATP hydrolysis can occur. (The mere presence of DNA is insufficient to activate ATP hydrolysis; SV40 form I or II DNA gives

*Dr. Paul Geshelin, personal communication.

Fig. 4. Co-sedimentation of exonuclease V and cross-linked T7 DNA on a sucrose gradient. The reaction mixture (0.1 ml) contained 10 mM Tris/HCl (pH 8), 10 mM MgCl₂, 1 mM ATP, 6 nmol of T7 [³²P] DNA (approximately 1 x 10⁶ cpm), and 8.8 units of exonuclease V. Incubation was at 37° for 2 min. The entire mixture was then loaded onto a sucrose gradient and centrifuged as described under "Experimental Procedure." Ten-drop fractions were collected from the bottom and assayed for [³²P] radioactivity, DNase activity, and ATPase activity. Five microliters was assayed directly for [³²P] radioactivity. DNase activity was measured by addition of 10 µl of a DNA mixture (30 mM MgCl₂, 0.5 mM ATP, 1.25 µCi of [³²P]DNA, 5 x 10⁶ cpm/mmol) to 20 µl of each fraction. After incubation at 37° for 30 min, 10 µl of each reaction was chromatographed for determination of acid-soluble [³²P] radioactivity. ATPase activity was measured by addition of 10 µl of an ATP mixture (30 mM MgCl₂, 0.5 mM ATP, 50 µCi/ml of [³²P]ATP) to 20 µl of each fraction. After incubation at 37° for 30 min, 5 µl of each reaction was chromatographed for determination of ATP and ADP.

Fig. 5. DNase and ATPase activities of exonuclease V on vaccinia DNA. Reaction mixture 1 (0.1 ml) contained 100 mM Tris/HCl, pH 8, 10 mM MgCl₂, 0.2 mM ATP, 1.25 µCi of [³²P]ATP, 1 nmol of vaccinia DNA, and 8.8 units of exonuclease V. Reactions 2 and 3 were identical except they contained either 1 nmol of cross-linked T7 DNA or 1 nmol of native T7 DNA. Incubation was at 37° for 30 min. At the indicated intervals, 5-µl aliquots were analyzed for per cent of ATP hydrolyzed. A duplicate reaction mixture 1 was also analyzed for digestion of vaccinia DNA by agarose gel electrophoresis of a 10-µl sample at each time point, staining of the gel with ethidium bromide, photographing with Polaroid P/N Type 55 film, and densitometric scanning of the band. Curves A, B, and C show ATP hydrolysis and Curve D shows vaccinia DNA digestion as determined from per cent decrease of fluorescence intensity in the DNA band.


**Hemophilus exo V Activity on Cross-linked DNA**

A. Vaccinia DNA

- Native structure
- Terminal cleavage
- Slow
- Fast
- Oligonucleotides

B. Psoralen-cross-linked DNA

- Exonuclease attack
- ATP
- ADP
- Activated ATPase complex

**Fig. 6.** Schematic illustrations of proposed mechanisms of *Hemophilus* exonuclease V activity on cross-linked DNA molecules. A, vaccinia terminal cross-links are represented as single-stranded loops that are cleaved endonucleolytically by exo V (represented as a shaded circle). B, psoralen cross-links are represented by transverse lines on the DNA. Exo V (shaded circles) digests the ends but remains bound at the cross-links and continuously hydrolyzes ATP.

no ATPase activation. However, exo V possesses weak single strand specific endonuclease activity (1, 5), and apparently it can slowly cleave the single-stranded terminal loops. As soon as one end of a molecule is opened by exo V digestion, the whole molecule is quickly digested to oligonucleotides with accompanying hydrolysis of ATP. Endonucleolytic terminal cleavage would appear to be the rate-limiting step.

Psoralen-cross-linked DNA behaves differently from vaccinia DNA as an exo V substrate. Psoralen cross-links are presumably introduced fairly randomly into T7 DNA so that, in general, they are situated internally leaving some portion of the ends free. It is reasonable to expect that exonuclease V can initiate attack at these ends without inhibition by cross-links several hundred bases toward the interior. Our experiments confirm this. Our heavily cross-linked DNA contains approximately 100 randomly intercalated psoralen molecules, leaving an estimated average of 2% psoralen-free DNA at the ends. This agrees well with the observed 2 to 3% available for digestion (Fig. 2, Curve A). With less extensively cross-linked DNA a greater average length at the ends of the DNA molecule is free of cross-links. We have observed with such DNA a proportionate increase in the amount of exo V digestible DNA (data not shown). Our various observations are most easily explained by the mechanism illustrated in Fig. 6B. An enzyme molecule binds to each end of a psoralen-cross-linked DNA molecule (10) and moves along cleaving phosphodiester bonds and hydrolyzing ATP. Progress is halted at the first cross-links encountered. At these sites enzyme remains tightly bound but continues to hydrolyze ATP. Regions of the molecule enclosed by cross-links form a resistant core because exo V can neither attack duplex DNA directly as an endonuclease (Fig. 1) nor bypass the cross-links.

The preincubation experiment of Fig. 3 and the co-sedimentation experiment of Fig. 4 provide evidence of a tight complex between psoralen-cross-linked DNA and exonuclease V. Since we have argued that the psoralen-free terminal region (2%) is not removed by exo V digestion, and that the enzyme cannot bypass a cross-link, we must conclude that the enzyme remains bound at the cross-link as illustrated in Fig. 6B. We have as yet been unable to demonstrate this by electron microscopy, nor have we ruled out dissociation of one or more enzyme subunits (1, 2). We have demonstrated that bound enzyme retains ATPase activity (Figs. 2 and 4), and that no subunit containing DNase activity is released by preincubation on psoralen-cross-linked DNA (Fig. 3). On the assumption that complete enzyme is bound at a cross-link, one must conclude that a firm stoichiometry between ATP hydrolysis and phosphodiester bond cleavage does not exist. Previously observed ratios of 30 to 40 ATP molecules hydrolyzed/DNA bond cleaved presumably do not reflect direct coupling between these two activities. In a subsequent paper (6), experiments are described that lend support to the hypothesis that ATP is utilized for movement and strand separation rather than for strand cleavage.

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