4426–4433 Nucleic Acids Research, 1995, Vol. 23, No. 21

A DNase from the trypanosomatid *Crithidia fasciculata*

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Received July 5, 1995; Revised and Accepted October 4, 1995

ABSTRACT

We have purified to homogeneity a DNase from a *Crithidia fasciculata* crude mitochondrial lysate. The enzyme is present in two forms, either as a 32 kDa polypeptide or as a multimer containing the 32 kDa polypeptide in association with a 56 kDa polypeptide. Native molecular weight measurements indicate that these forms are a monomer and possibly an $\alpha_2\beta_2$ tetramer, respectively. The monomeric and multimeric forms of the enzyme are similar in their catalytic activities. Both digest double-stranded DNA about twice as efficiently as single-stranded DNA. They introduce single-strand breaks into a supercoiled plasmid but do not efficiently make double-strand breaks. They degrade a linearized plasmid more efficiently than a nicked plasmid. Both enzymes degrade a 5′-^{32}P-labeled double-stranded oligonucleotide to completion, with the 5′-terminal nucleotide ultimately being released as a 5′-mononucleotide. One difference between the monomeric and multimeric forms of the enzyme, demonstrated by a band shift assay, is that the multimeric form binds tightly to double-stranded DNA, possibly aggregating it.

INTRODUCTION

Trypanosomatids are protozoan parasites which cause important diseases in humans and livestock. They are also significant because they have unusual biological properties. One of their most interesting features is their mitochondrial DNA, known as kinetoplast DNA (kDNA), which consists of several thousand DNA rings which are topologically interlocked (see refs 1–5 for reviews on kDNA). Our laboratory has had a long interest in studying the structure and replication of kDNA, and most of our studies have focused on *Crithidia fasciculata*, a parasite of insects, and the African trypanosomes. One of our goals is to study proteins and enzymes which may be involved in the maintenance and replication of kDNA. This is a relatively unexplored area; only a few proteins and enzymes which clearly are associated with kDNA have been isolated and studied.

We recently set out to purify a DNA ligase, an enzyme presumably involved in kDNA replication, from a crude mitochondrial fraction. During several stages of this purification the ligase co-fractionated with a powerful DNase activity. We decided to purify this nuclease to homogeneity, and here we describe some of its properties.

MATERIALS AND METHODS

Nucleic acids

For uniform ^{3}H-labeling of *Escherichia coli* chromosomal DNA, *E.coli* DH5a was grown at 37°C in 500 ml minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NH_4Cl, 0.5 g NaCl and 3 mg $CaCl_2$/l) supplemented with 0.5% casein hydrolysate and 0.2% glycerol. [^{3}H]thymidine (5 μCi/ml) was added to the culture at early log phase (OD_{600} = 0.2). The cells were harvested at mid log phase (OD_{600} = 0.6) and lysed by adding SDS to a concentration of 0.5% and proteinase K to 500 μg/ml. After 1 h at 37°C the lysate was extracted with chloroform/isoamyl alcohol (24:1) and nucleic acids were precipitated with ethanol. After dissolving in 10 ml of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA (TE) buffer, RNase A was added to 50 μg/ml and the sample was incubated at 37°C for 1 h. Proteinase K (10 μg/ml) was then added and incubated at 35°C for 60 min. The DNA was extracted with phenol and then with chloroform/isoamyl alcohol (24:1). Finally it was ethanol precipitated and resuspended in 5 ml TE buffer (0.3 mg/ml, 20 000 c.p.m./μg). Plasmid pPK201/CAT (6) was labeled under the same conditions and purified using Qiagen plasmid preparation kits. It was stored in TE buffer at 25 μg/ml, and its specific activity was 20 000 c.p.m./μg. The plasmid was linearized with restriction enzyme *Pvu*I. Linearized plasmid was then ^{32}P-labeled at its 5′ end with T4 polynucleotide kinase or at its 3′-end with Klenow polymerase using standard protocols.

DNase assays

All DNase assays were performed in a 25 μl reaction containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 5% glycerol, DNA substrate, and enzyme. Incubation was at 35°C for the designated time. Three types of DNA substrates were used in this study. In one, the substrate was composed of a 30mer (3′-AATCAGTCGGGATACTTGTCGTTGTTGTGA) annealed to two complementary 15mer oligonucleotides (5′-TTAGTCAGCCCTATG and 5′-[^{32}P]AACAGCAACAACACT). This substrate was initially prepared for the DNA ligase assay. Other substrates were uniformly ^{3}H-labeled supercoiled or linearized plasmid pPK 201/CAT and *E.coli* chromosomal DNA. In some cases the *E.coli* DNA was denatured by boiling for 10 min followed by quick cooling on ice. To quantitate the acid solubility of the *E.coli* DNA substrate, the reaction was terminated

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by chilling and the addition of BSA to 1 mg/ml and trichloroacetic acid to 10%. Acid-soluble radioactivity was measured by scintillation counting of the supernatant. With double-stranded *E.coli* DNA, one unit of activity is defined as the amount required to produce 1 pmol of total nucleotide in acid-soluble form in 30 min at 35°C. The assay is linear with enzyme concentration in the range of 0–0.8 U.

Gel electrophoresis

DNA was electrophoresed in 1% agarose gels in 45 mM Tris-borate, pH 8.0, 1 mM EDTA (TBE) buffer containing 0.1 μg/ml ethidium bromide. For fluorography, the gels were treated with En^{3}Hance (New England Nuclear) and then dried under vacuum. Kodak X-Omat AR film was used for fluorography after preflashing. The film was exposed at –70°C for 3–7 days.

Large scale growth of *C.fasciculata*

Parasites were grown in a Fermatron fermenter (New Brunswick Scientific Co. Inc.) containing 150 l of medium [1.8% Deltown AE80M peptone, 0.45% yeast extract, 0.45% NaCl, 0.9% glucose and 10 mg/l of Hemin (7)]. The cells were grown at 26°C with aeration for 24 h to a density of ~3×10^7/ml and then harvested in a Sharples continuous flow centrifuge. The cells were washed in STE (250 mM sucrose, 50 mM Tris–HCl, pH 7.5, 1 mM EDTA) and then centrifuged (5000 *g*, 10 min, 4°C). The yield was ~560 g of cells (wet weight).

Preparation of the mitochondrial lysate

Cells were disrupted at 4°C in a Parr cell disruption bomb according to the manufacturer's instructions. Washed cells (280 g) were resuspended at a concentration of 1 g/5 ml in cell disruption buffer (50 mM Tris–HCl, pH 7.5, 250 mM sucrose, 1 mM EDTA, 5 mM $MgCl_2$, 50 mM KCl, 1 μg/ml leupeptin) and transferred to the bomb. The bomb was pressurized with nitrogen to 1800–2000 p.s.i. for 30 min, while stirring with a magnetic bar at the bottom of the bomb. The cells were disrupted as the bomb was released to atmospheric pressure through the discharge valve. Pressure usually dropped during the discharge of the cells. It was therefore necessary to stop the flow, increase the nitrogen pressure to 1800–2000 p.s.i., and then resume the discharge. When examined by light and electron microscopy, at least 98% of the cells were disrupted and the kDNA appeared in its characteristic condensed form. To prepare crude mitochondria, the cell homogenate was centrifuged in an SS-34 rotor at 13 000 r.p.m. for 20 min or a GS-3 rotor at 9000 r.p.m. for 20 min and the supernatant was discarded. The pellet was resuspended in cell disruption buffer and centrifuged again under the same conditions. Mitochondria were lysed by gentle stirring at 4°C for 30 min in mitochondrial lysis buffer [0.5 M KCl, 0.25% (v/v) NP-40, 2 mM EDTA, 10% (v/v) glycerol, 25 mM Tris–HCl (pH 7.5), 2 μg/ml leupeptin]. Debris from the mitochondrial lysate was removed by centrifugation (27 000 *g*, 30 min, 4°C).

Production of antibody

Antibody to the *C.fasciculata* nuclease was prepared by immunization of BALB/c mice. Mice were immunized by intraperitoneal injection with 2–4 μg of highly purified protein (Fraction 8) every 2 weeks for 8 weeks. The initial inoculation was prepared with Freund's complete adjuvant, and subsequent boosts were in Freund's incomplete adjuvant. The antiserum diluted 1/1000 recognizes the 32 kDa nuclease on Western blots.

RESULTS

Purification of the DNase activity

This procedure was originally optimized for purification of a DNA ligase, but the nuclease activity co-fractionated closely with the ligase until the last purification step on Phenyl Superose FPLC. Except where indicated, all purification procedures were conducted at 4°C using Buffer A (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, 1 μg/ml leupeptin), and all dialyses were performed against Buffer A at 4°C. Assays during purification utilized *E.coli* double-stranded [^{3}H]DNA as a substrate. Table 1 presents a summary of the purification.

Table 1. Purification of the *C.fasciculata* DNase. The starting material was a lysate of a crude mitochondrial fraction derived from 280 g of log phase *C.fasciculata*

Fraction		Protein[a] (mg)	Activity[b] (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
1.	Mitochondrial lysate	30100	3120	0.10	1	100
2.	PEG supernatant	21200	2650	0.13	1.3	85
3.	Q-Sepharose	2780	2310	0.83	8.3	74
4.	Bio-Gel HT	945	2440	2.6	26	78
5.	DNA–cellulose	158	1460	9.2	9.2	47
6.	Mono S	32	799	25	250	26
7.	Phenyl Superose I[c]	0.66	254	385	3850	8
8.	Phenyl Superose II[c]	0.42	307	730	7300	10

[a]Protein was assayed with the Coomassie Protein Assay Reagent (Pierce) according to the manufacturer's instruction.
[b]Activity was assayed using double-stranded *E.coli* [^{3}H]DNA as substrate.
[c]Fractions 7 and 8 are multimeric and monomeric forms of the enzyme, respectively, both derived from Fraction 6. They have a combined yield of 18%.

Crude mitochondrial lysate (Fraction 1, 700 ml, see Materials and Methods), prepared from ~280 g cells, was adjusted to 2 M KCl and 5% polyethylene glycol (PEG 8000, Sigma) (by addition of solid reagents) with gentle stirring for 1 h at 4°C. The mixture was then centrifuged at 27 500 *g* at 4°C for 30 min. The supernatant was collected and dialyzed exhaustively against Buffer A (Fraction 2). Fraction 2 (600 ml) was loaded onto a 100 ml Q-Sepharose column (Pharmacia) at a flow rate of 2 ml/min. The column was washed with 1 l Buffer A containing 0.2 M KCl. The nuclease activity was eluted with 500 ml Buffer A containing 0.6 M KCl (Fraction 3). This was directly loaded onto a hydroxylapatite column (BioRad Bio-Gel HT; 5 × 15 cm) at 1 ml/min, which then was eluted with a 1200 ml linear gradient from 0 to 1 M K_2HPO_4 (pH 9.0). The activity eluted between 0.3 and 0.5 M, and the active fractions were pooled (160 ml) and dialyzed (Fraction 4). Fraction 4 was loaded onto a 20 ml double-stranded DNA–cellulose column (Pharmacia) at 0.5 ml/min and the column was washed with 100 ml of Buffer A. The column was eluted with a 150 ml gradient of 50 mM to 1 M KCl in Buffer A. The DNase activity appeared between 0.25 and 0.5 M (Fraction 5, 40 ml). After dialysis, 40 ml of Fraction 5 was loaded onto a Mono S FPLC column (Pharmacia, HR5/5) at 0.25 ml/min. The column was eluted with a 20 ml gradient of 50 mM–1 M KCl in buffer A. In this column the activity clearly eluted in two peaks, one at ~0.3 M KCl and the second at 0.5 M KCl. The active fractions were combined (Fraction 6), dialyzed, adjusted with 2.5 M K_2HPO_4 (pH 9.0) to a final concentration of 1 M, and loaded onto a Phenyl Superose FPLC column (Pharmacia, HR5/5) at a flow rate of 0.25 ml/min. The column was washed with 5 ml 1 M K_2HPO_4 and eluted with a 30 ml linear reverse gradient from 1 to 0 M K_2HPO_4. The DNase activity was recovered as two peaks, designated Fractions 7 and 8, between ~0.7 and 0.5 M salt. These highly purified proteins were dialyzed against Buffer A containing 0.1 M KCl and stored at –70°C.

Both Fractions 7 and 8 are fairly stable under our storage conditions, maintaining >50% of activity after >6 months. Starting from the crude mitochondrial lysate supernatant, the DNase was purified ~7300-fold for Fraction 8 and 3850-fold for Fraction 7 (Table 1). The yield from 280 g of cells was ~0.7 mg of Fraction 7 and 0.4 mg of Fraction 8.

SDS–PAGE analysis of purification fractions

Figure 1A shows an analysis of Fractions 1–8 by SDS–PAGE and silver staining. Fraction 8 is a homogeneous protein of 32 kDa. Fraction 7 contains two polypeptides, the 32 kDa protein (lower arrow on right of panel A) and another component of 56 kDa (upper arrow on right of panel A). We will demonstrate below that the two polypeptides in Fraction 7 constitute a multimeric complex. Since silver staining may not provide an accurate indication of the relative amounts of the two polypeptides in the complex, we stained Fraction 7, which had been further purified by glycerol gradient centrifugation (not shown), with Coomassie Blue (Fig. 1B). The relative staining of the two bands suggests that the two polypeptides are present in roughly equimolar quantities.

Evidence that the DNase activity is associated with the 32 kDa polypeptide

We assayed DNase activity (using double- and single-stranded *E.coli* [^{3}H]DNA as substrates) and protein composition (by SDS–PAGE) in fractions eluted from the Phenyl-Superose FPLC column. As shown in Figure 2, the activity of the enzyme correlates closely with the concentration of 32 kDa protein in each fraction. We found a similar correlation for the nicking of supercoiled plasmid DNA or the degradation of ^{32}P-labeled oligonucleotide substrate (see Fig. 5). There was also co-fractionation of the activity and 32 kDa protein on the Mono S column and in a glycerol gradient fractionation (not shown), providing conclusive evidence that the 32 kDa polypeptide is responsible for DNase activity. In addition, in most purification steps for the multimeric form of the enzyme (Fraction 7), we observed co-fractionation of the 32 kDa and the 56 kDa polypeptides. However, when Fraction 7 was re-run on a Phenyl Superose FPLC column, there was partial separation of the 56 kDa protein and the 32 kDa protein.

It is important to note that a mouse antibody, prepared against purified Fraction 8, also reacted in a Western blot with the 32 kDa polypeptide in Fraction 7 (not shown). Therefore, we conclude that the same 32 kDa protein is present in both fractions.

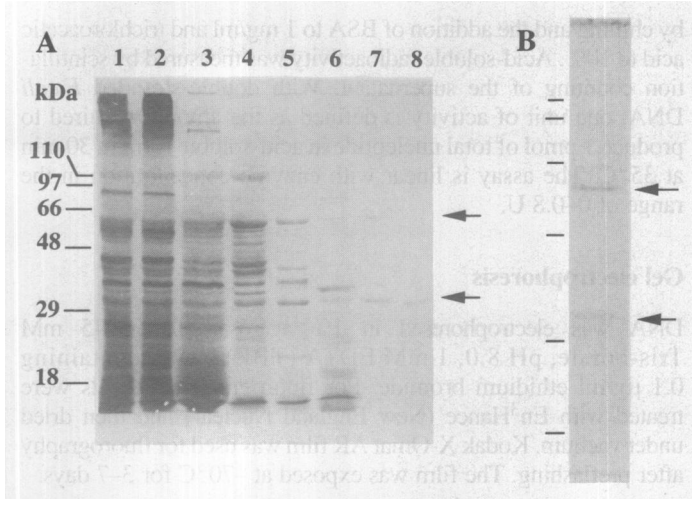

Figure 1. SDS–PAGE analysis of nuclease purification. (**A**) Aliquots of each fraction were electrophoresed on an 11% SDS–polyacrylamide gel which was then silver stained. Lane 1, Fraction 1, crude mitochondrial lysate, 5 µg protein; lane 2, Fraction 2, PEG supernatant, 3 µg; lane 3, Fraction 3, Q-Sepharose, 2 µg; lane 4, Fraction 4, Bio-Gel HT, 2 µg; lane 5, Fraction 5, DNA–cellulose, 1.5 µg; lane 6, Fraction 6, Mono S, 1.5 µg; lane 7, Fraction 7, Phenyl Superose I, 0.3 µg; lane 8, Fraction 8, Phenyl Superose II, 0.1 µg. (**B**) Coomassie stained gel of a sample of Fraction 7 which had been further purified by glycerol gradient centrifugation. The arrows on the right of each panel indicate the 32 kDa and the 56 kDa polypeptides. The scale on the left of (B) corresponds to the size markers shown in (A).

Hydrodynamic properties and subunit structure of the two forms of the DNase

To assess the enzyme's size and subunit composition, we subjected samples of Fractions 7 and 8 to glycerol gradient centrifugation. We assayed the gradient fractions for nuclease activity using *E.coli* [^{3}H]DNA as a substrate (Fig. 3A). We used SDS–PAGE to evaluate the sedimentation of three reference proteins, of known $s_{20,w}$, in a parallel gradient. As determined by linear interpolation, the DNase activity in Fractions 7 and 8 had $s_{20,w}$ values of 7 S and 3.5 S, respectively.

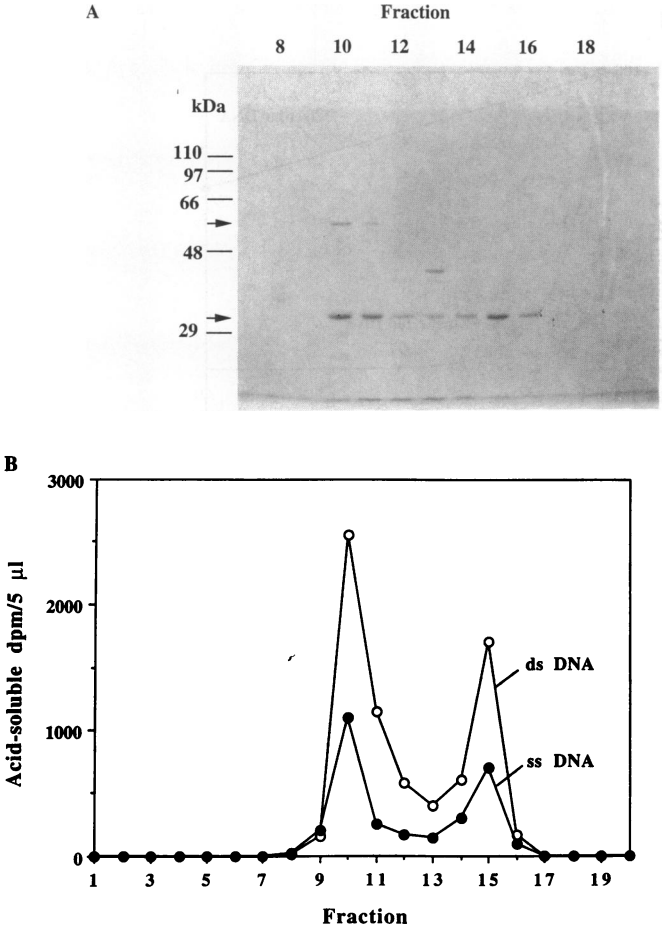

Figure 2. DNase activity co-fractionates with the 32 kDa polypeptide. Aliquots from the Phenyl Superose fractions were analyzed by SDS–PAGE and for DNase activity. (**A**) SDS–polyacrylamide gel of 5 µl of each fraction; the gel was stained with silver. The lower arrow indicates the 32 kDa polypeptide which corresponds to the DNase activity; the upper arrow indicates the 56 kDa polypeptide. (**B**) DNase activity, on 5 µl of each fraction, using either double-stranded (open circles) or single-stranded (closed circles) *E.coli* [^{3}H]DNA as a substrate.

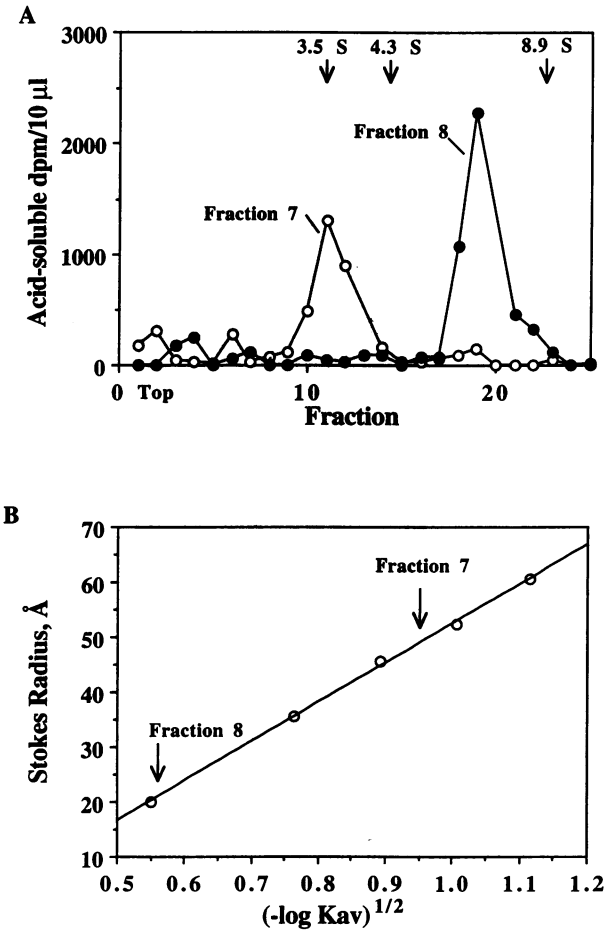

Figure 3. Hydrodynamic properties of Fractions 7 and 8. (**A**) 200 µl aliquots of Fractions 7 (open circles) and 8 (closed circles) were centrifuged on separate 5 ml preformed 10–35% glycerol gradients in 50 mM Tris–HCl, pH 7.5, 150 mM KCl, 2 mM DTT and 1 mM EDTA. Centrifugation was carried out in a SW55 Ti rotor at 50 000 r.p.m. for 36 h at 4°C. Standards were bovine erythrocyte carbonic anhydrase (3.8 S), BSA (4.3 S) and sweet potato β-amylase (8.9 S) which were centrifuged in a parallel gradient. After centrifugation, 25 fractions were collected from each gradient by puncturing the bottom of the tube. The nuclease assay (10 µl aliquots) used double-stranded *E.coli* DNA as substrate. The standard proteins were assayed by SDS–PAGE. (**B**) The Stokes' radii were estimated by gel filtration at 4°C on a Superose 12 FPLC column (30 ml bed volume) with the column equilibrated in Buffer A. Standards were horse spleen apoferritin, bovine liver catalase, yeast alcohol dehydrogenase, BSA and bovine erythrocyte carbonic anhydrase. These proteins have Stokes' radii of 60.6, 52.3, 46, 35.6 and 20.1 , respectively. All standard proteins are from Sigma.

We next determined the Stokes' radius of Fractions 7 and 8 using gel filtration on a 30-ml Superose 12 column. The eluted proteins were detected by UV absorption (280 nm) and assayed by SDS–PAGE. By comparison with protein standards, the Stokes' radii of Fraction 7 and Fraction 8 are 51 and 23Å, respectively (Fig. 3B). All of the hydrodynamic properties of the two proteins are presented in Table 2. From these values we used the Svedberg equation (8) to calculate the native molecular weights of the Fraction 7 and 8 enzymes. These values are 147 000 and 32 000, respectively. It is likely, therefore, that Fraction 8 is a monomer. Since the two subunits of Fraction 7 appear to be equimolar (Fig. 1B), it is likely that it is an $\alpha_2\beta_2$ tetramer consisting of two 32 kDa and two 56 kDa subunits. However, the data are also consistant with a trimeric structure, containing either two 56 kDa subunits and one 32 kDa, or alternatively, one 56 kDa and two 32 kDa subunits.

The multimeric structure of the DNase was confirmed by immuno-precipitation experiments. Antibody produced against the 32 kDa polypeptide was incubated with mitochondrial lysate and then Protein A–Sepharose was added to precipitate the antigen–antibody complexes. The 56 kDa polypeptide co-precipitated with the 32 kDa polypeptide as judged by SDS–PAGE (data not shown).

Enzymatic properties of the DNase

As shown in Figure 2, both the multimeric form (Fraction 7) and the monomeric form (Fraction 8) of the DNase were active against both double- and single-stranded *E.coli* [^{3}H]DNA, with activity on the double-stranded substrate about twice that on the single-stranded. Both forms of the enzyme have similar specific activities with respect to the 32 kDa polypeptide.

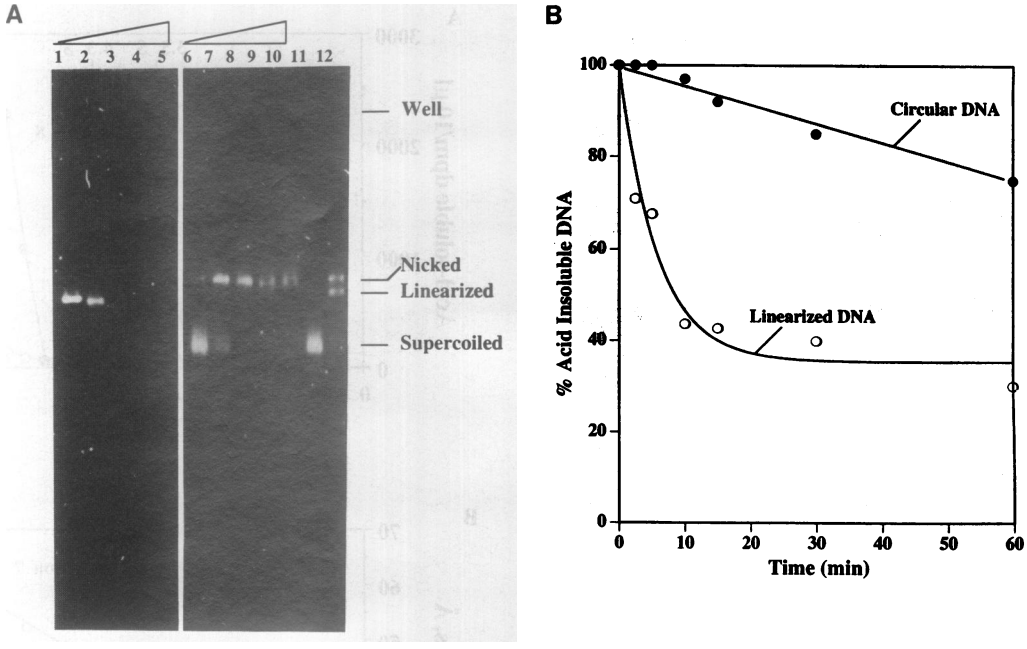

Figure 4. Comparison of linearized and supercoiled DNA as substrates for the *C.fasciculata* DNase. The substrate (0.5 µg) was plasmid pPK201/CAT (6) which was either supercoiled (lanes 6–10) or linearized by *Pvu*I digestion (lanes 1–5). (**A**) The DNase products were analyzed by 1% agarose gel electrophoresis and ethidium staining. The reactions were carried out at 35°C for 1 h. The amount of enzyme was 0 (lanes 1 and 6), 0.05 µg (lanes 2 and 7), 0.125 µg (lanes 3 and 8), 0.25 µg (lanes 4 and 9) and 0.5 µg (lanes 5 and 10), respectively. Lanes 11 and 12 contain markers. (**B**) A time course of the nuclease reaction, under the same conditions as in (A), using ^{3}H-labeled pPK201/CAT as a substrate and 0.05 µg enzyme. After incubation, the acid-soluble supernatant was counted in scintillation fluid.

Table 2. Hydrodynamic properties of the *C.fasciculata* nuclease

Protein fraction	Stokes' radius (Å)	Sedimentation coefficient ($s_{20,w}$)	f/fo	Molecular weight (SDS–PAGE)	Molecular weight (native)
Fraction 8	23	3.5	1.10	32 000	32 000
Fraction 7	51	7.0	1.47	32 000, 56 000	147 000

Stokes' radii and sedimentation coefficients were determined in Figure 3. The native molecular weight and f/fo were calculated from the Svedberg equation using 0.725 cm^3/g as the partial specific volume (8). Values for the subunit molecular weight (SDS–PAGE) are from Figure 1.

To determine whether the purified nuclease has the ability to cleave a DNA substrate at internal positions, we used a ^{3}H-labeled supercoiled plasmid as substrate. In a titration of Fraction 8 enzyme, there is a conversion of the supercoiled substrate to a nicked product (Fig. 4A, lanes 6–10). There was little conversion to a linearized form of the substrate, indicating that the enzyme makes few double-strand breaks. The same was true for Fraction 7 enzyme (not shown). Under identical conditions, we also used the same plasmid which had been linearized with a restriction enzyme (Fig. 4A, lanes 1–5). In this case the DNA was more efficiently degraded than with the supercoiled substrate, indicating a preference for degradation of a linear molecule. We confirmed this finding by measuring a time course of the production of acid-insoluble radioactivity from the same substrates (Fig. 4B). Again, the linearized DNA was degraded much more rapidly.

We next assayed the Phenyl Superose fractions (the same fractions as used in Fig. 2) using a 5′-^{32}P-labeled oligonucleotide as substrate. The products were analyzed on a 20% sequencing gel (Fig. 5). Comparison of the relative activity with the SDS–PAGE analysis of the same fractions (Fig. 2A) indicates that the activity correlates fairly closely with the presence of the 32 kDa polypeptide. With each form of the enzyme a ladder of products is formed, containing ~14 smaller fragments. When the digestion is allowed to proceed to near completion, the major product is [5′-^{32}P]dAMP derived from the 5′ terminus. On PEI-cellulose TLC analysis, this product co-migrated with a 5′ dAMP standard (data not shown).

We also used as a substrate a linearized ^{3}H-labeled plasmid DNA which had been labeled with ^{32}P either at its 3′ ends or 5′ ends. We digested each substrate with Fraction 8 enzyme and analyzed the acid-soluble radioactivity. Figure 6 shows the kinetics of conversion to acid solubility of both radiolabels.

The DNase activity in both Fraction 7 and Fraction 8 requires a divalent cation with optimal activity being obtained with 5 mM $MgCl_2$. At 2.5 mM the activity was reduced by ~40% and at 10 mM it was reduced by 20%. Ca^{2+} cannot replace the Mg^{2+} and fails to activate the DNase activity. EDTA inhibits the enzyme activity when added in excess of the divalent cation. The DNase activity is inhibited by KCl at relative low concentration (~50% inhibition at 75 mM for Fraction 8 and 100 mM for Fraction 7). ATP at 1 mM had no detectable effect upon the DNase activity.

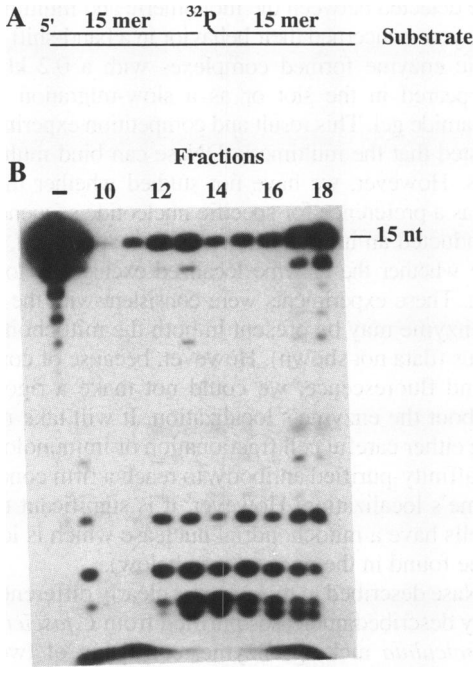

Figure 5. Activity on an oligonucleotide substrate. Aliquots from the Phenyl Superose fractions (the same as in Fig. 2) were analyzed for nuclease activity using a 5′ ^{32}P-labeled DNA oligonucleotide. (**A**) Diagram of the oligonucleotide, which contains one 30mer and two 15mers (see Materials and Methods for sequence). To form this substrate, the three oligonucleotides (each at 0.2 µM in 25 mM Tris–HCl, pH 7.5, 1 mM EDTA) were heated to 100°C and then cooled to room temperature over a period of 1 h. (**B**) DNase assay on 5 µl fractions of the Phenyl Superose column. After incubation at 35°C for 30 min, 5 µl of each sample was mixed with 5 µl sequencing gel buffer and loaded onto a 20% sequencing gel. The gel was autoradiographed. The sample to the left of Fraction 9 is the starting substrate.

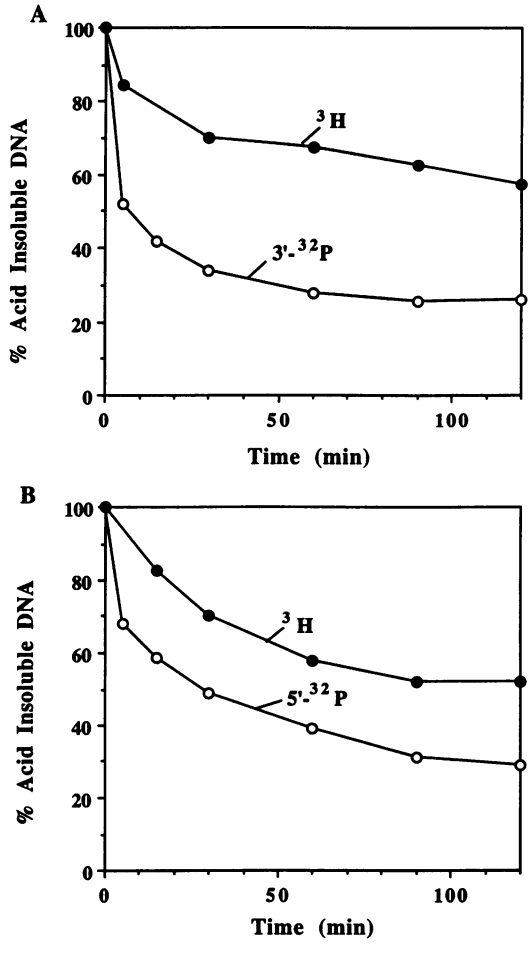

Figure 6. Degradation of ^{3}H-labeled pPK201/CAT labeled at the 3′ (**A**) or 5′ end (**B**) with ^{32}P. The substrate (0.3 µg) was incubated at 35°C with 0.1 µg of Fraction 7 DNase. Acid-soluble radioactivity was measured in a scintillation counter.

The DNase activity did not change between pH 6.5 and 8.5 using Tris–HCl or Bis-Tris–HCl buffer.

Binding of the DNase to a double-stranded DNA fragment

We used a band shift assay to measure the efficiency of binding of the enzyme to a ^{32}P-labeled 210 bp restriction fragment. To minimize degradation of the substrate, we carried out the binding reactions on ice for 10 min. Under these conditions, as assayed by TCA precipitation, <10% of the substrate was converted into an acid-soluble form (data not shown). As shown in Figure 7A, Fraction 8 (the monomeric enzyme, lanes 3 and 6) caused no significant band shift. In contrast, Fraction 7 (the multimer) formed two complexes in the presence of Mg^{2+} (lane 2) but none in the absence of Mg^{2+} (lane 5). One complex (I) remained in the gel slot and another (II) migrated a short distance into the gel. This result suggested that the multimer may efficiently bind two or more substrate molecules, possibly forming aggregates. Complex II may contain two substrate molecules linked to one multimer and complex I may contain multiple substrate and multiple enzyme molecules. ATP did not affect the binding activity of the DNase. To evaluate this binding reaction further, we used a non-radioactive DNA oligonucleotide (20 bases) as a competitive substrate. As shown in Figure 7B, a third protein–DNA complex appeared (complex III) with either a single- or double-stranded oligonucleotide. It is reasonable to assume that complex III contains an enzyme multimer bound to one 210 bp fragment and one oligonucleotide.

DISCUSSION

Using a mitochondrial fraction of *C.fasciculata,* we have purified to virtual homogeneity two forms of a DNase. This enzyme apparently exists both as a monomeric 32 kDa protein (Fraction 8) and a multimeric form which contains roughly equimolar quantities of the 32 kDa polypeptide and a 56 kDa polypeptide (Fraction 7) (Fig. 1). The multimer has a native molecular weight of 147 000, consistant with either a tetramer with an $\alpha_2\beta_2$ structure or a trimeric structure. The 32 kDa polypeptide is responsible for catalytic activity.

The substrate specificity of the two forms of the enzyme appears to be very similar. Both degrade double-stranded DNA about twice as fast as single-stranded DNA (Fig. 2B) and the rate depends upon the concentration of the 32 kDa polypeptide. The enzyme has endonucleolytic activity, as shown by the introduction of nicks into a supercoiled plasmid (Fig. 4A, lanes 6–10). However, a comparison of the degradation of linearized and

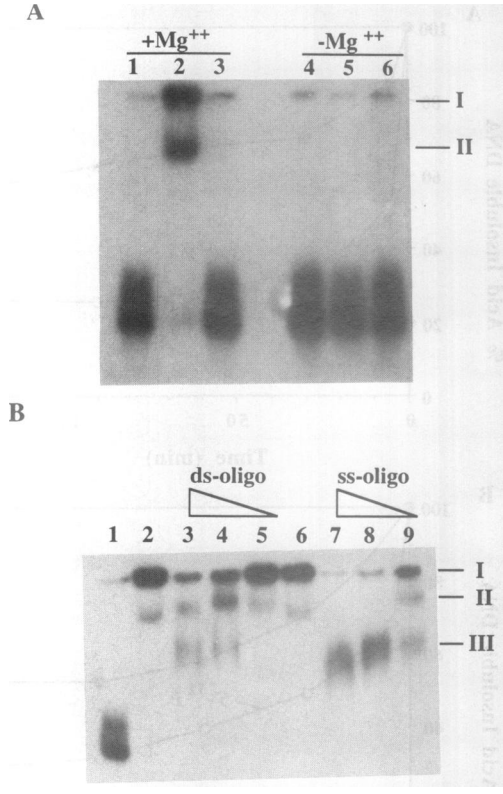

Figure 7. Binding of a DNA fragment by Fractions 7 and 8. A 210 bp fragment containing the *C.fasciculata* kDNA minicircle conserved sequence [nucleotides 275–485 (22)] was excised from pDP312 plasmid by *Bam*HI/*Eco*RI digestion and end-labeled with [α-^{32}P]dATP using DNA polymerase. Labeled DNA fragment (~0.1 μg) and 50 ng of enzyme were used in each reaction. The buffer contained 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 10% glycerol, with or without 5 mM $MgCl_2$. The binding reaction was carried out on ice for 10 min and then the reaction mixture was loaded on a 5% polyacrylamide gel in 0.8× TBE buffer. After running at 100 V for 2 h, the gel was autoradiographed. (**A**) DNA binding assay with (lanes 1–3) or without (lanes 4–6) $MgCl_2$. Lanes 1 and 4, no protein added; lanes 2 and 5, fraction 7; lanes 3 and 6, fraction 8. (**B**) DNA binding assay with competitive DNA oligonucleotides. The double-stranded ^{32}P-labeled substrate (0.1 μg) was mixed with either a single-stranded oligonucleotide (5′AAGGGGTTGGTGTAATACAG) or its double-stranded form. The underlined sequence of this oligonucleotide corresponded to nucleotides 1740–1751 in the minicircle sequence (22). Lane 1, no protein added; lanes 2 and 6, 50 ng of Fraction 7; lanes 3, 4 and 5, 50 ng of Fraction 7 with 10, 1, and 0.2 ng of double-stranded oligonucleotide, respectively; lane 7, 8 and 9, 50 ng of Fraction 7 with 5, 0.5 and 0.1 ng of single-stranded oligonucleotide, respectively.

supercoiled plasmid indicates a preference for the linearized species (Fig. 4B, and compare lanes 1–5 and 6–10 in Fig. 4A). This finding suggests that the enzyme may have an exonuclease component. The production of a ladder of products from a 5′ ^{32}P-labeled double-stranded oligonucleotide is consistent with a 3′ exonuclease activity, but these products could be equally well explained by a random endonuclease activity. We therefore tested Fraction 8 enzyme on a linearized ^{3}H-labeled plasmid containing a ^{32}P-label at either its 3′ or 5′ ends. Both ^{32}P-labels were acid-solubilized more efficiently than the ^{3}H, but the release of ^{32}P was not as selective as expected if this enzyme behaved predominantly as an exonuclease. There is little difference between the substrates labeled at the 3′ and 5′ ends. It is likely, therefore, that this enzyme is an endonuclease which has some preference for attacking near the ends of a linear molecule. The only difference which we detected between the monomeric and multimeric forms of this enzyme concerned their behavior in a band-shift assay. The multimeric enzyme formed complexes with a 0.2 kb substrate which appeared in the slot or as a slow-migration band in a polyacrylamide gel. This result and competition experiments (Fig. 7) suggested that the multimeric DNase can bind multiple DNA molecules. However, we have not studied whether this binding activity has a preference for specific nucleotide sequences.

We conducted an immunofluorescence experiment, hoping to determine whether the enzyme localized exclusively to the mitochondrion. These experiments were consistent with the possibility that the enzyme may be present in both the mitochondrion and the nucleus (data not shown). However, because of considerable background fluorescence, we could not make a rigorous conclusion about the enzyme's localization. It will take more data, involving either careful cell fractionation or immunolocalization using an affinity-purified antibody, to reach a firm conclusion on the enzyme's localization. However, it is significant that mammalian cells have a mitochondrial nuclease which is identical to an enzyme found in the nucleus (see below).

The DNase described in this paper is clearly different from two previously described nucleases purified from *C.fasciculata*. One is a *C.fasciculata* nicking enzyme, consisting of two 60 kDa subunits, which prefers a bent DNA substrate (9–11). The other is endonuclease A, an enzyme of ~120 kDa which introduces nicks at limited sites in a double-stranded DNA substrate (12). Neither enzyme has been localized intracellularly. From their catalytic and physical properties, the enzymes reported in this paper differ from those described previously.

It is of interest to compare this *C.fasciculata* nuclease with mitochondrial nucleases from other species. Mitochondrial enzymes have been purified from many species, including mammals (13), *Drosophila melanogaster* (14), *Saccharomyces cerevisiae* (15) and *Neurospora crassa* (16). The function of these enzymes is not known, and a yeast mutant lacking the mitochondrial nuclease is phenotypically identical to wild-type (17). These enzymes have differences in molecular weight and substrate specificity, although the yeast and mammalian enzymes have sequence homology (18,19). The mammalian enzyme has a catalytic subunit of 29 kDa, similar in size to that of the *C.fasciculata* DNase, although it is homodimeric in structure. Surprisingly, the mammalian mitochondrial nuclease is identical to Endonuclease G, an enzyme found in the nucleus (18,20). The mammalian enzyme is striking in having a strong preference for cleaving within a sequence of 12 consecutive guanine residues that is found within the mitochondrial D-loop region (21). The mouse mitochondrial nuclease (23) has some similarities to the *C.fasciculata* DNase, although it prefers a single-stranded substrate; however, its specific activity is several orders of magnitude higher than that of the *C.fasciculata* DNase.

Further characterization of the substrate specificity of the *C.fasciculata* DNase and analysis of its amino sequence will be required before it can be compared more meaningfully with these other mitochondrial enzymes. At this time the function of the *C.fasciculata* DNase is unknown.

ACKNOWLEDGEMENTS

This work was supported by Grant GM 27608 from the National Institutes of Health and a grant from the MacArthur Foundation. We thank members of our laboratory for many enlightening

discussions. We also thank Scott Morrow for his assistance with large scale growth of *C.fasciculata.*

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