Volume 5 Number 8 August 1978 Nucleic Acids Research

Purification of a DNA nicking-closing enzyme from mouse L cells

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Received 20 April 1978

ABSTRACT

A DNA nicking-closing enzyme has been purified from the nuclei of mouse L cells to 90% homogeneity. The denatured and reduced form of the enzyme has a molecular weight of 68,000 which is in agreement with the molecular weight of the native enzyme as determined by gel filtration and by sucrose sedimentation velocity assuming the protein is globular. Therefore, the active form of the enzyme is a monopolypeptide. Its isoelectric point is pH 4.2 + 0.2. The nicking-closing activity does not require a cofactor and does not involve any sulfhydryl group. The enzyme requires 0.2 M NaCl and pH in the range of 6.5-7.5 for optimal activity.

INTRODUCTION

Torsional stress in the DNA duplex may be relieved by a process of nicking, swivelling, and resealing the DNA. This overall process can be catalyzed by one protein, the DNA nicking-closing enzyme. A nicked intermediate with the enzyme covalently attached has been shown to occur in the reaction catalyzed by the rat liver enzyme (1,2). Although closed circular DNA has been used to assay for the nicking-closing activity, supercoiling of the substrate has been shown not to be required for activity of the mouse enzyme (3). By inference, torsional stress in both linear and closed circular DNA may be relieved by the same enzyme.

The nicking-closing activity appears to be ubiquitous in nature. It was first demonstrated in E. coli (4) and subsequently in eukaryotes such as rat liver (5), human KB cells (6), fertilized Drosophila eggs (7), calf thymus (8), Hela and mouse L cells (9,10), and recently, in vaccinia virus (11). Nicking-closing enzymes have been purified from rat liver cells and human KB cells. By studying the sedimentation of the native proteins and the gel electrophoresis of the SDS denatured proteins, these two enzymes were shown to be single polypeptides with molecular weights of 66,000 (12) and 60,000 (6) respectively. By contrast, other studies indicated that the mouse enzyme consisted of two similar subunits each with a

molecular weight of about 37,000 (9); later studies suggested that both the mouse and HeLa enzymes were either histone H1 or somehow tightly associated with it (10,13). I show here that the mouse enzyme is not similar to histone H1, that it is a rather acidic protein, and that its molecular weight (68,000) is similar to that of the rat liver and the human KB cell enzymes.

MATERIALS AND METHODS

PM2 DNA I and PM2 ^{3}H DNA I, prepared as in Espejo and Canelo (14), were generously provided by R. Watson and R. Parker. ^{125}I-plasminogen from dog serum was a gift of J. Tobler. Other reagents and their sources are as follows: bovine serum albumin, ovalbumin, chymotrypsinogen and "Ultrapure" sucrose (for all density gradients) from Schwarz-Mann; conalbumin from Sigma; poly(ethylene glycol), Carbowax 6000 from Union Carbide; ampholytes from LKB Instruments (pH 3.5-10) and from Brinkmann Instruments (pH 3-5); hydroxylapatite (Bio-Gel HTP) and all reagents for polyacrylamide gel electrophoresis from Bio-Rad; sodium p-(hydroxymercuri)-benzoate from Aldrich Chemical; N-ethylmaleimide from Eastman Kodak and from Calbiochem; fluorascamine from Roche Diagnostics; carrier free Na^{125}I from ICN; chloramine-T and sodium metabisulfite from Matheson Coleman and Bell; Seakem agarose from Marine Colloids; Aquasol from New England Nuclear.

Growth of Cells

LA9 cells were grown in 10 liter suspensions of Eagle's medium (Dulbecco's modification) with 10% calf serum. The cells were harvested at a density of 5 x 10^{5} cells/ml.

Nicking-Closing Enzyme Assay

The assay, which is similar to that previously described (6,11), is based on the decreased uptake of ethidium bromide by closed circular DNA when the number of superhelical turns is decreased by enzyme activity. There is a corresponding increase in the electrophoretic mobility of DNA I_o. The reaction mixture (100 µl) contained 0.2M NaCl, 0.02 M potassium phosphate (pH 7.3), 2 x 10^{-4} M EDTA, 100 µg/ml BSA, 1 µg of PM2 ^{3}H DNA I (2 x 10^{3} cpm/µg), and enzyme. The enzyme was diluted in 0.2 M NaCl, 0.02 M potassium phosphate (pH 7.3), and 100 µg/ml BSA. Reaction mixtures were incubated at 37^{o} for 30 min after which the reaction was stopped by adding SDS to 0.1% and chilling to 0^{o}. 20 µl aliquots of the reaction mixtures were then electrophoresed on a 1% agarose slab gel containing ethidium bromide as in Pulleyblank *et al.* (3). The slab gel was illuminated with

short wavelength ultraviolet light, and photographed with Kodak Plus-X film (3). The bands corresponding to DNA I and I_o were sliced out of the agarose gel and each slice incubated with 12 ml of Aquasol at 37°, 8 hours. Afterward, the samples were counted using a Beckman LS-230 scintillation spectrometer. One unit of activity is defined as the amount of enzyme coverting half of 1.0 µg of PM2 ^{3}H DNA I to I_o in 30 min.

Spin Dialysis

Samples were desalted by centrifuging through Sephadex G-25 as in Neal and Florini (15).

Isoelectric Focusing

Focusing was performed as in Press and Klinman (16). 1 ml Tuberculin disposable syringes, used as focusing tubes, were suspended between two vertical buffer tanks. The syringe tips were covered with dialysis membranes each held in place by a circular tygon strip and a rubber band. A sucrose gradient was generated by layering 0.25 ml of 50%, 40%, 30%, 20%, and 10% sucrose into each tube. This gradient contained half of the total ampholines in the 50% sucrose layer to ensure higher conductivity. In wide range focusing, 2% w/v of LKB pH 3.5-10 ampholines were used. In narrow range focusing, a mixture of 1.64% w/v Buchmann pH 3-5 ampholines and 0.36% w/v pH 3.5-10 was used. The anode solution was 1.5% H_3PO_4 and the cathode solution was 10% 2-aminoethanol. The bottom tank solution contained 50% sucrose.

An enzyme sample was desalted by spin dialysis, mixed with a sucrose layer, and introduced into the tube before focusing. The sucrose layer selected was removed from the final isoelectric position so as to require the nicking-closing enzyme to migrate from the position of insertion in order to focus. Isoelectric focusing at 4° was initiated at 200 V. The current was maintained at about 0.2 mA per tube which required an increase of about 40 V every 30 min. Upon reaching 360 V, the focusing was continued at this voltage for 11 hours. Under these conditions, two mixtures of myoglobin, cytochrome c, and hemoglobin placed at the acidic and basic sucrose layers separated and focused after 6 hours at 360 V. The steady state end point of 11 hour for focusing the nicking-closing enzyme was determined by analyzing the activity profile at several time points. After focusing, the top tank was drained, the Tygon ring was removed from each tip and replaced by a serum stopper. Each tube was harvested by puncturing with a 22 gauge needle. Fractions were collected at 4° to maintain the enzymatic activity.

pH Determination

Each 50 µl fraction from an isoelectric focusing gradient was diluted into 1 ml 0.1 M KCl, and the pH determined using a Radiometer Model PHM 64 pH meter. Since the focusing was at 4^{o}, the pH was determined also at 4^{o}.

Iodination of Proteins

Isofocused samples were dialyzed against 1 M NaCl, 0.05 M sodium phosphate (pH 7.6) for 8 hours, and against 0.05 M sodium phosphate (pH 7.6) for 8 hours. Dialysis against NaCl removed ampholines which otherwise competed with proteins during iodination. Proteins were iodinated by a modification of the chloramine-T method (17). 1 mCi of carrier free $Na^{125}I$ was added to 10 ng of protein in 200 µl of 0.05 M sodium phosphate (pH 7.6). The reaction was initiated by addition of 5 µl of 1 mg/ml chloramine-T, and allowed to proceed for 70 sec, 20^{o}. Then 5 µl of 2 mg/ml sodium metabisulfite was added to stop the reaction. The unbound ^{125}I was removed by spin dialysis.

SDS-Polyacrylamide Gel Electrophoresis

Protein samples were denatured and reduced in Laemmli "sample buffers" and electrophoresed in a 10% Laemmli slab gel (18). Molecular weight standards were electrophoresed in an accompanying slot of the same slab gel. Gels were stained and fixed in Coomassie blue, 10% acetic acid, 10% methanol. Destaining was in 10% acetic acid and 10% methanol.

Autoradiography

After fixing, the slab gel was dried down on a gel dryer (Hoeffer Scientific) and Kodak No Screen X-Ray film was placed over the dried gel. The major and minor protein bands were cut out of the dried gel and counted using a Beckman Biogamma II spectrometer.

Protein Determination

Protein concentration was determined by the method of Lowry et al. (19) except for the PEG supernatant (Fraction II) and the Sephadex G-150 filtrate (Fraction V). For the PEG fraction, the method of xylene brilliant cyanin G staining (20) was used. For the Sephadex fraction, the fluorescamine method of protein determination (21) was used. Both methods were calibrated using dilutions of standard concentrations of BSA determined by the Lowry method.

Velocity Sedimentation

Linear gradients of 5-20% sucrose contained 0.5 M KCl, 0.02 M potassium phosphate (pH 7.3), 1 mM EDTA, 0.1 mM PMSF. Centrifugation was at 40,000 rpm at 4^{o} for 22 hours in a SW 50.1 rotor. Sedimentation markers

were present in all gradients and included plasminogen, BSA, and ovalbumin. 84 µl fractions were collected at 4°.

RESULTS

Purification of DNA Nicking-Closing Enzyme

All steps were performed at 0-4°. They are summarized in Table I.

Isolation of Nuclei and Preparation of Extract

LA9 cell nuclei were prepared as in Vosberg and Vinograd (10) except for two modifications: firstly, 0.1 mM PMSF was present in all buffers; secondly, cells were swollen and homogenized in 1 mM $CaCl_2$ which resulted in a higher yield of intact nuclei. 100 ml of packed nuclei were isolated from 128 g of LA9 cells.

The nuclei were resuspended in 360 ml of 0.02 M potassium phosphate (pH 7.3), 0.05 M KCl, 0.1 mM PMSF (Buffer A) containing 1 mM 2-mercaptoethanol and homogenized in a Sorvall omnimixer at 0.75 maximal speed for 45 sec. Solid potassium chloride was added to bring the homogenate to 1 M salt. The viscous homogenate was sonically irradiated in five batches in a stainless steel beaker at 0° for a total of 2.5 min. This was done in five 30 sec bursts with 1 min cooling periods. The nuclear debris was removed by centrifugation in a Beckman Ty35 rotor, 20,000 rpm for 5 hours. The supernatant was Fraction I.

Poly(ethylene glycol) Precipitation

Solid PEG was added to 5% w/v and the mixture stirred for 25 min to dissolve the PEG completely. Precipitation was allowed to proceed for 12 hours. After this period, a white precipitate was collected by centri-

TABLE I

Purification of Mouse Cell Nicking-Closing Enzyme*

	Fraction	Volume ml	Activity units	Protein mg	Specific Activity units/mg	Yield %
I.	Nuclear extract	484	10×10^7	1,800	5.5×10^4	100
II.	PEG precipitation	480	8.2×10^7	540	1.5×10^5	82
III.	Hydroxylapatite A	120	5.4×10^7	3.7	1.4×10^7	54
IV.	Hydroxylapatite B	7.4	2.3×10^7	1.0	2.3×10^7	23
V.	Sephadex G-150	26	1.9×10^7	6.7×10^{-2}	2.8×10^8	19

*Prepared from 5×10^{10} nuclei.

fugation at 12,000 g for 30 min in a Sorvall refrigerated centrifuge. The clear yellow supernatant was Fraction II.

Hydroxylapatite Chromatography A

Fraction II was applied immediately to a hydroxylapatite column (4.9 cm^2 x 35 cm) equilibrated with Buffer A containing 1 M KCl and 1 mM 2-mercaptoethanol. The column was washed with 880 ml of the same buffer, and then with 840 ml of 0.2 M potassium phosphate (pH 7.3), 1 mM 2-mercaptoethanol in Buffer A. A 1 liter gradient from 0.2 to 1.0 M potassium phosphate (pH 7.3) in Buffer A with 1 mM 2-mercaptoethanol was applied to the column (Fig. 1). Fractions containing the activity eluted between 0.47 and 0.56 M potassium phosphate and they were pooled to give Fraction III.

Hydroxylapatite Chromatography B

Fraction III (132 ml) was diluted to 1,200 ml with 1 M KCl in Buffer A, and applied to a 4.9 cm^2 x 2 cm hydroxylapatite column. Again, the

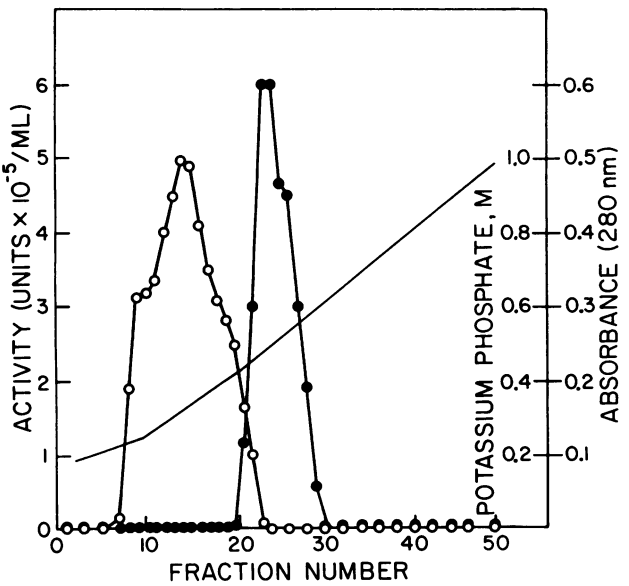

Fig. 1. Hydroxylapatite chromatography of nicking-closing enzyme.

●, nicking-closing activity (units x 10^{-5}/ml); o, absorbance at 280 nm; —, concentration of potassium phosphate (pH 7.3). Fraction II was applied to the column and the column was washed with 880 ml 1 M KCl, 0.02 M potassium phosphate (pH 7.3), 0.1 mM PMSF, 1 mM 2-mercaptoethanol. The column was then washed with 800 ml 0.2 M potassium phosphate (pH 7.3), 0.05 M KCl, 0.1 mM PMSF, 1 mM 2-mercaptoethanol. This procedure eluted 99% of the protein and none of the activity (data not shown). A linear gradient of 0.20 to 1.00 M potassium phosphate in Buffer A was then applied to the column. Fractions of 20 ml were collected. The activity eluted between 0.47 to 0.56 M potassium phosphate.

column was washed successively with Buffer A (100 ml) containing 1 M KCl and Buffer A (30 ml) containing 0.2 M potassium phosphate (pH 7.3). Then Buffer A containing 0.7 M potassium phosphate (pH 7.3) was applied to the column, and the activity was eluted in 7.4 ml (Fraction IV).

Sephadex G-150 Chromatography

6.5 ml of Fraction IV was filtered through a 4.9 cm^2 x 80 cm column of Sephadex G-150 equilibrated with 0.2 M KCl, 20 mM potassium phosphate (pH7.3), 0.1 mM PMSF (Fig. 2). Fractions of 6.5 ml were collected. The column was equilibrated by fractionating a mixture of dextran blue, ^{125}I plasminogen, conalbumin, BSA, and ovalbumin. The activity eluted from fraction 34 to 37. These fractions were made 1 M NaCl, 10% glycerol, and stored at -20° until needed (Fraction V). In 1 M NaCl at 4°, Fraction V was stable for 1 month. At -20° and in 1 M NaCl and 10% glycerol, Fraction V retained at least 80% of its activity over 7 months.

HeLa Enzyme Preparation

26 ml of packed nuclei were isolated from 30 g of HeLa cells, and the

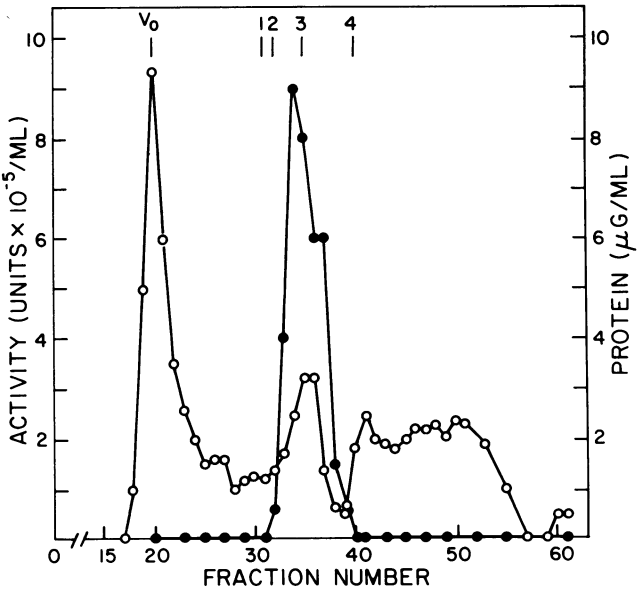

Fig. 2. Sephadex G-150 gel chromatography. ●, nicking-closing activity (units x 10^{-5}/ml); o, protein (μg/ml) as determined by the fluorascamine method. 6.5 ml of Fraction IV was loaded on the 4.9 cm^2 x 80 cm column and eluted with 0.2 M KCl, 0.02 M potassium phosphate (pH 7.3), 0.1 mM PMSF at a flow rate of 26 ml/hour. Fractions of 6.5 ml were collected. The column was calibrated by fractionating a mixture of blue dextran (V_o), ^{125}I plasminogen (1), conalbumin (2), BSA (3), and ovalbumin (4). The included volume was 263 ml.

nicking-closing enzyme purified through the hydroxylapatite A step (Fraction III). The elution profile of the hydroxylapatite step was very similar to that observed with the enzyme from LA9, and the activity also eluted between 0.47 and 0.56 M potassium phosphate (pH 7.3).

Requirements for Activity

The optimal KCl or NaCl concentration for activity was 0.2 M. No sharp pH dependence was found; rather, a range of pH 6.5-7.5 gave optimal activity. At the standard assay NaCl concentration of 0.2 M, no nuclease activity could be detected. No nuclease activity could be detected on lowering the NaCl to 0.04 M and adding $MgCl_2$ to 4 mM.

As shown in Table II, N-ethylmaleimide, a specific sulfhydryl reagent, did not inhibit the enzyme, and 2-mercaptoethanol did not stimulate the activity. When 2-mercaptoethanol was omitted throughout the purification an equivalent yield of activity was obtained at Fraction V. p-(Hydroxymercuri)-benzoate did inhibit completely at 0.4 mM. At this concentration, this reagent reacts with other amino acid side chains in addition to SH; presumably, some such reaction caused the inhibition.

No cofactor was necessary for Fraction V. A boiled aliquot of Fraction I, when added to Fraction V, did not stimulate the activity.

TABLE II

Requirements for Activity

Standard Assay Condition with 1 Unit of Fraction V

and 1 μg PM2 DNA I

Conditions	DNA I_o Formed μg	Relative Activity %
Complete mix	0.50	100
Omit NaCl	0.00	0
Omit NaCl, add 0.2 M pot. phosphate (pH 7.3)	0.05	10
Omit BSA, add NEM (2 mM)	0.50	100
Omit BSA, add PCMB (0.4 mM)	0.00	0
Add 2-mercaptoethanol (1 mM)	0.50	100
Add Triton (0.1%)	0.50	100
Add SDS (0.1%)	0.00	0
Add boiled Fraction I	0.45	90

Physical Properties

Isoelectric Point

For analysis of subunit structure, samples of each of fractions 34 to 37 from Sephadex G-150 filtration were purified further by isoelectric focusing. Up to 280 µl containing 2.8 x 10^4 units from each fraction were spin dialyzed twice to reduce the salt to less than 5 mM. The dialysate was mixed immediately with sucrose, pH 3-5 ampholines and Triton X-100, and focused for 13 hours as in "Materials and Methods". After focusing, the tube was punctured at the bottom and 50 µl fractions were collected. Aliquots were diluted with enzyme dilution buffer containing 0.1% Triton, and assayed for nicking-closing activity. The most active fractions were dialyzed against 1 M NaCl to remove ampholines (see "Materials and Methods"), and then iodinated for SDS-polyacrylamide gel electrophoresis. The activity recovered after spin dialyzing the enzyme was 50%, and from spin dialyzing and focusing 12%. 0.1% Triton increased the recovery of activity from focusing by approximately two-fold. Insufficient protein was present to allow quantitation by either the Lowry or the fluorascamine method.

The nicking-closing enzyme focused at pH 4.0-4.7 in wide range focusing (not shown). This isoelectric range was obtained with the anode either at the top electrophoresis tank or at the bottom tank; in either case, the enzyme was required to migrate toward its pI. In the narrow range focusing (Fig. 3) the isoelectric point was determined to be pH 4.2 $\pm$ 0.2 at 4°. The isoelectric point was not altered by 0.1% Triton.

Molecular Weight and Purity

The pool of active fractions from isoelectric focusing was examined by slab SDS-polyacrylamide gel electrophoresis. Since no bands were detectable by Coomassie blue staining, the material was iodinated before electrophoresis. Autoradiography of the slab gel revealed a single major protein band comprising 90% of the iodinated proteins. As shown in Fig. 4, isoelectric focusing of two different active fractions of Sephadex G-150 filtrate yielded an identical major band which comigrated with BSA. Channel a resulted from the focusing of the active Sephadex fraction 34; channel b resulted from the focusing of the active fraction 37. Since the molecular weights of the minor bands were different in focusing different Sephadex fractions, and since the specific activity of the enzyme was similar in both cases, the major band was taken to be the nicking-closing enzyme. Migration in the same slab gel of iodinated marker proteins allowed an estimate of 68,000 for the molecular weight of the denatured and reduced enzyme (Fig. 5). The

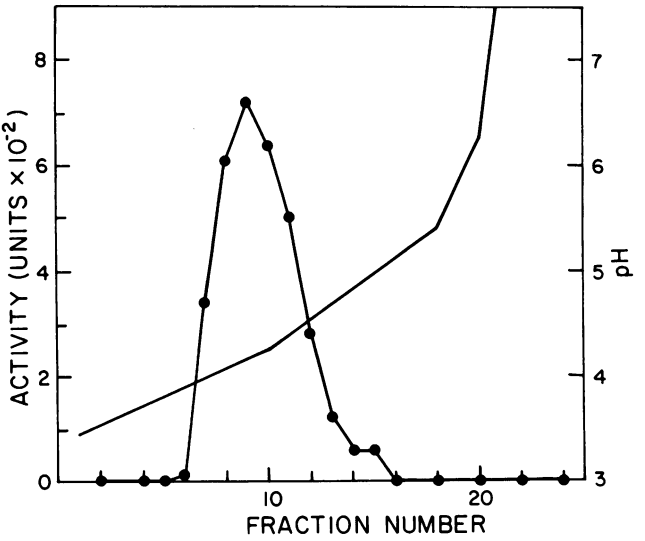

Fig. 3. Isoelectric focusing of the nicking-closing enzyme. ●, nicking-closing activity (units x 10^{-3}); —, pH. The activity peak from the Sephadex filtrate (28,000 units) was focused at 4° in a 1.25 ml sucrose gradient with pH 3-5 ampholines and 0.1% Triton (see "Materials and Methods"). After 13 hours focusing, 50 µl fractions were collected at 4°, and assayed immediately for DNA nicking-closing activity.

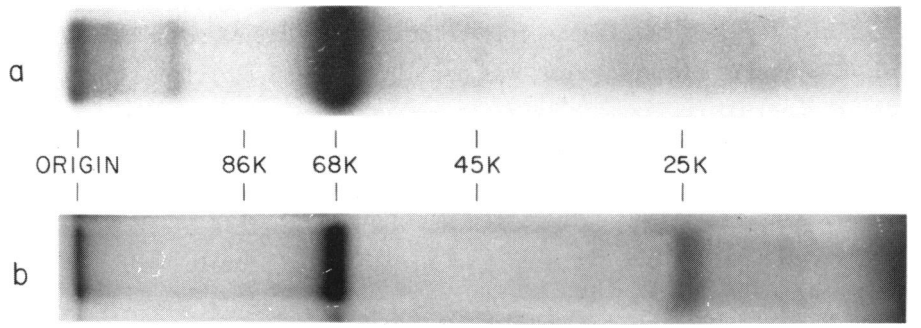

Fig. 4. SDS-polyacrylamide gel electrophoresis of the nicking-closing enzyme. The activity peak from isoelectric focusing was iodinated, denatured and reduced, and electrophoresed on a Laemmli slab gel as in "Materials and Methods." Channel a is the activity peak from the focusing of Sephadex fraction 34; channel b is from the focusing of Sephadex fraction 37. Iodinated marker proteins conalbumin, BSA, ovalbumin, and chymotrypsinogen were electrophoresed on the same slab gel. These had previously been mixedwith the same set of unlabeled proteins and electrophoresed. The slab gel was Coomasie blue stained, dried down, and autoradiographed. The mobility of each of the iodinated bands corresponded to each of the stained bands. In the two channels each major band was overexposed to show the minor bands.

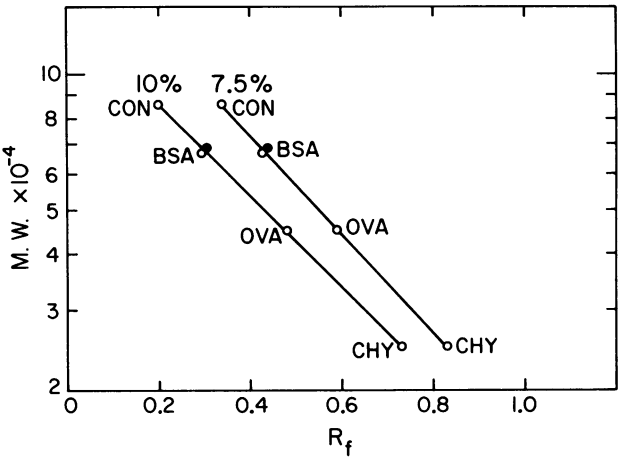

Fig. 5. The molecular weight of the denatured and reduced nicking-closing enzyme was determined by comparing its electrophoretic mobility (R_f) with those of protein standards, conalbumin (CON), BSA, ovalbumin (OVA), chymotrypsinogen (CHY).

same estimate was obtained in 7.5% and 10% gels.

The molecular weight of the native enzyme was estimated by Sephadex gel filtration (22) and sucrose velocity sedimentation (23). The elution volume of the nicking-closing activity in reference to marker proteins (Fig. 2) allowed a calculation of the K_{av} which was consistent with a molecular weight of 72,000 for a globular protein. In sucrose gradient sedimentation (Fig. 6), the activity moved slightly behind BSA, and had a sedimentation coefficient of 4.1s. If one assumes that the enzyme and the markers are spherical, and applies the equation (23) $(M_1/M_2) = (S_1/S_2)^{3/2}$, one calculates a molecular weight of 61,000 for the enzyme. These two estimates for the native protein together with the SDS gel molecular weight indicate that the native enzyme is a monopolypeptide.

Removal of Histone H1 Contamination

No histone H1 was detected after isoelectric focusing, unlike the nicking-closing activity reported by Vosberg and Vinograd (10). A longer time of precipitation of the nuclear extract with 5% PEG had removed the H1. A nuclear extract was divided into two equal portions at the Fraction I stage. One portion underwent a 25 min PEG precipitation and the other portion underwent a 12 hour precipitation. Both portions were chromatographed on parallel hydroxylapatite columns to yield their respective Fraction III. Since Fraction II contained a substantial number of major

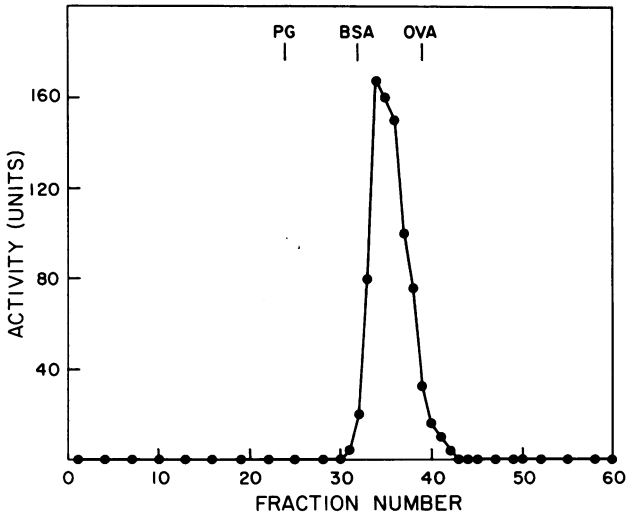

Fig. 6. Sucrose sedimentation velocity. 70 µl containing 3,000 units of Fraction V and marker proteins was sedimented through a preformed 5-20% linear sucrose gradient in 0.5 M KCl, 0.02 M potassium phosphate (pH 7.3), 1 mM EDTA, and 0.1 mM PMSF. Centrifugation was in a SW50.1 rotor at 40,000 rpm for 22 hours at 4°. Fractions of 84 µl were collected at 4°. The markers were canine serum plasminogen, BSA, and ovalbumin.

proteins, the two preparations of Fraction III were compared. Fig. 7 compares the SDS polyacrylamide gels of the active pools from hydroxylapatite A (Fig. 1). Channel a corresponds to the preparation obtained after a 25 min PEG precipitation. This preparation, after a DEAE cellulose column and a further hydroxylapatite column, would lead to the major H1 band reported by Vosberg and Vinograd (10, 13). Channel b corresponds to the preparation after a 12 hour PEG precipitation. This preparation was free of H1 and showed a substantial amount of high molecular weight proteins. Furthermore, both preparations yielded the same total units of activity in the PEG supernatant (Fraction II) and in the subsequent hydroxylapatite active peak (Fraction III). However, the longer PEG precipitation led to a nine-fold higher specific activity for Fraction III (1.45×10^7 u/mg).

A similar absence of H1 was noted in the SDS-polyacrylamide gel of the HeLa hydroxylapatite pool (Fraction III). This pool contained 70% of the nicking-closing activity of the sonicated HeLa nuclei and had a specific activity of 8.5×10^6 u/mg.

DISCUSSION

In the enzyme purification procedure, a longer period of standing in

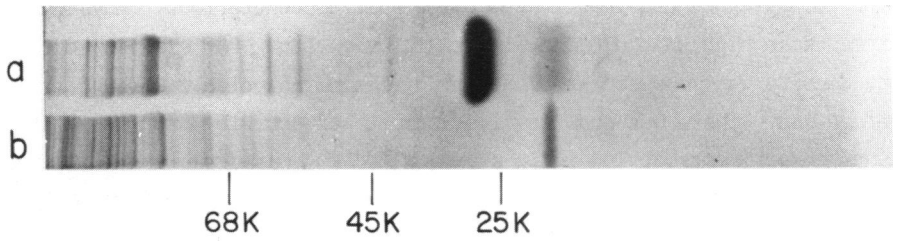

Fig. 7. SDS-polyacrylamide gel electrophoresis of Fraction III. Parallel enzyme preparations were made with (a) 25 min PEG precipitation and (b) 12 hour PEG precipitation. The two supernatants were loaded onto two equivalent hydroxylapatite columns, washed, and eluted as in "Results." Each peak activity eluting at ~0.5 M potassium phosphate (pH7.3) was electrophoresed on a slab gel with marker proteins in an adjacent slot. Coomassie blue staining showed (a) to be contaminated with histone H1 and (b) to be H1 free. Both preparations had the same total units of activity. The major band in (a) comigrated with H1 standard in acid-urea and basic gels.

5% PEG than used previously resulted in the precipitation of histone H1 either as histone or in a complex with DNA. The resulting supernatant retained all of the enzyme activity. The purified enzyme has a molecular weight of 68,000 and an isoelectric point of pH 4.2 $\pm$ 0.2. Therefore histone H1 is not an integral part of the nicking-closing activity.

A previous study (3) showed that supercoiling of the closed circular DNA substrate is not required for the action of the mouse nicking-closing enzyme. The study first demonstrated that the enzyme converted closed circular DNA into a limit product set of topological isomers having a mean degree of supercoiling of ~0. The isomers differed from each other by single superhelical turns, and their relative masses fit a Boltzmann distribution. The study then showed that by re-reacting each isomer with the enzyme, the original distribution was regenerated. Therefore supercoiling of the DNA was not required for enzyme activity. This conclusion is still valid even though we now know that a H1 contaminated nicking-closing activity was used. The reason is that histone H1 did not alter the duplex winding number of closed circular DNA under the reaction condition of 0.2 M NaCl. This was shown in the same study since polynucleotide ligase acting on nicked circular DNA generated, under the same conditions, a set of topological isomers indistinguishable from that generated by the H1 contaminated nicking-closing activity acting on closed circular DNA.

Throughout the enzyme purification, it was noticed that the activity was stable in the presence of high concentrations of NaCl. The enzyme

appeared to aggregate and inactivate at low salt concentrations. Physical measurements in high salt did not lead to inactivation. For example, the buoyant density was measured as 1.28 g/ml by cesium chloride equilibrium centrifugation. There was quantitative recovery of activity from the CsCl gradient. Experiments at low ionic strength led to considerable inactivation. For example, attempts to recover nicking-closing activity from native polyacrylamide gels failed. The extremely low ionic strength of the isoelectric focusing may be responsible for the poor recovery of activity. However, the ampholines, acting as zwitterions, may have counteracted the enzyme's aggregation. The two-fold increased recovery by focusing in 0.1% Triton supports the hypothesis of aggregation.

Even though only 12% of the input activity was recovered after isoelectric focusing, no activity migrated out of the pH gradient (i.e. no missing component was observed). The activity profiles of several equivalent focusings were monitored as a function of focusing time, and only one active component was observed. It is not too surprising to find an acidic isoelectric point for an enzyme such as the nicking-closing enzyme that acts on DNA; for example, E. coli DNA polymerase I has a pI of 5.2 (24), Drosophila DNA polymerase a pI of 5.3 (24), and HeLa RNA polymerase II a pI of 4.7 (25).

Assuming that the proteins of Fraction V have an average molecular weight of 70,000, the activity observed (Table I) under the standard assay condition indicates that over a thousand PM2 DNA I can be relaxed to I_o by one protein molecule. This is a much higher ratio than the 20 PM2 DNA I relaxed per protein molecule observed by Vosberg and Vinograd (10) due to the H1 contamination in their final product. Whether the enzyme relaxes DNA I in one hit or multiple steps is not known, but the overall reaction is catalytic.

By physical methods, the mouse nicking-closing enzyme is shown to be a monopolypeptide with a molecular weight of 68,000. This is in good agreement with the molecular weights of 60,000 (6) and 66,000 (12) for the human KB cell enzyme and the rat liver enzyme respectively. That a similar enzymatic activity is performed by a monopolypeptide of similar molecular weight suggests that the mammalian nicking-closing enzyme is a conserved protein. The role of this enzyme in DNA replication, in DNA recombination, in RNA transcription, or in other processes where local relief of a torsional constraint on duplex DNA is needed is not known.

The abbreviations used are: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PEG, poly(ethylene glycol); PMSF, α-Toluene sulfonyl fluoride DNA I, native closed circular DNA; DNA I_o, closed circular DNA with approximately zero superhelical turns, the end product of treating DNA I with the nicking-closing enzyme.

ACKNOWLEDGEMENTS

This work was supported by Grants CA 08014 and GM 15327 from the United States Public Health Service to Dr. Jerome Vinograd. Thanks are due to Judith Campbell, Norman Davidson, and Tom Maniatis for their critical review of the manuscript.

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