PURIFICATION AND DEMONSTRATION OF THE ENZYMATIC CHARACTER OF THE NICKING-CLOSING PROTEIN FROM MOUSE L CELLS

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SUMMARY: A rapid procedure for the purification of the nicking-closing enzyme from mouse L cells is described. The procedure reproducibly provides high yields of enzyme. A purity of greater than 90% is obtained in five steps. The enzymatic character of the nicking-closing activity has been demonstrated. On the average 20 PM2 DNA I molecules are completely relaxed by one enzyme molecule. The enzyme releases superhelical turns from closed circular DNA by providing a swivel through a sequence of successive nicking and closing events. It is not known yet whether the release of superhelical turns proceeds via a one hit or a multiple hit mechanism.

Nicking-closing enzymes (N-C enzymes)* capable of releasing superhelical turns in closed circular DNA appear to be ubiquitous in nature (1-5). Postulated *in vivo* functions for these enzymes include the introduction of transient swivels ahead of the growing replication forks as well as possible involvements in transcription and condensation and decondensation in chromatin.

We have previously purified and partially characterized this enzyme (5). While the protein was apparently homogeneous in gel electrophoresis experiments, the yield was low and the amounts were inadequate for detailed studies. We have, therefore, revised the purification procedure, which is now simple and reproducibly provides good yields of enzyme. A detailed description of the new method is described here, together with evidence that demonstrates the N-C protein acts enzymatically rather than stoichiometrically. A preliminary report of this work has been communicated (6).

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*We previously referred to the N-C enzyme as "relaxation protein" (5). The newer term has the advantage that it describes the activity on non-supercoiled as well as on supercoiled circular DNA (7).

Abbreviations: N-C enzyme, nicking-closing enzyme; PEG, polyethyleneglycol; EtdBr, ethidium bromide; SB, standard buffer; MET, β-mercaptoethanol.

MATERIALS AND METHODS

Materials: LA9 cells were grown in 1 ℓ suspension cultures in Dulbecco's modification of Eagle's medium supplemented with 8% calf serum. Spinners were inoculated at 7 to 8 x 10^4 cells/ml and harvested after 68 hours at approximately 5 x 10^5 cells/ml. PM2 DNA isolated from phage particles (8) was used as a substrate for the N-C enzyme. Polyethyleneglycol 6000 (PEG) was obtained from Union Carbide. Hydroxyapatite (Biogel HTP) was purchased from Bio-Rad. DEAE cellulose (type DE 52) was from Whatman (England). Agarose was from Marine Colloids Inc. and CsCl was from Reliable Chemical Company. Ethidium bromide (EtdBr) was obtained from Calbiochem. Bovine serum albumin, fraction V (crystalline) was from Miles Laboratories.
Standard reaction conditions: One µg of PM2 DNA I was incubated in 8 ml glass serum tubes in a total volume of 250 µl containing 10 mM potassium phosphate, pH 7.0, 0.2 M NaCl, 0.2 mM EDTA, 1 mM spermidine, 50 µg/ml bovine serum albumin and N-C enzyme in 1 to 15 µl for the centrifugation and fluorometric assays. For gel electrophoresis in 0.8% agarose slab gels, the minimum reaction volume, 50 µl, contained 50 ng of PM2 DNA I in the above solution. After addition of 2.5 µl of bromophenol blue in 50% glycerol, 25 µl from each assay were loaded onto the gel.
Assay methods: Three different assay methods have been used for the analysis of the products of the relaxation process and for monitoring the activity during the purification. These are: centrifugation in EtdBr-CsCl density gradients, changes in binding of EtdBr assayed fluorometrically and gel electrophoresis in agarose gels. The methods are described elsewhere (5,6). The activity in crude lysates was determined by the first method and in column eluates by the third method. The second method was used for the quantification of the N-C enzyme activity. Alternative procedures using this technique for the measurement of nicking-closing activities have been independently developed by Morgan and Pulleyblank (9) and Champoux and Durnford (10).

RESULTS

1. Purification of N-C enzyme: All purification steps were carried out at 0 to 4°. The standard buffer (SB) was 0.02 M potassium phosphate, pH 7.0, and 1 mM β-mercaptoethanol (MET). All other potassium phosphate buffers were also at pH 7.0 and contained 1 mM MET. Dialysis steps were avoided throughout the procedure.

Nuclear extracts: Cells were harvested in a RC 2B Sorvall centrifuge using the continuous flow system of Szent-Gyorgyi and Blum. The cells (16 ml packed volume from eight 1 ℓ cultures) were washed once and immediately suspended in 60 ml of SB to allow swelling during a 10 min. incubation in ice. 30 ml cell portions were disrupted with 8-10 strokes of a tight fitting pestle in a Dounce homogenizer. 20 ml of 1 M sucrose (in 0.01 M Tris pH 7.6 and 1 mM EDTA) were added, and the nuclei pelleted by centrifugation in an IEC centrifuge for 5 min. at 2,500 rev/min. The nuclear pellets were

suspended in 60 ml of SB and gently shaken without attempting to disperse clumped nuclei. The centrifugation procedure was repeated twice or until the supernatant was no longer turbid. The nuclei were resuspended in 60 ml of SB and disrupted in a Sorvall Omnimix at 0.75 of the maximal speed for 45 seconds. Solid KCl and SB were added to bring the suspension to 100 ml and 1 M KCl, and gently stirred for 15-20 min. until the KCl dissolved in the viscous suspension.

The combination of the high salt concentration and sonication yielded complete solubilization of the enzyme, as compared with 25% upon sonication in SB and 25% upon treatment with high salt. The viscous lysate was sonicated in a 6.5 cm wide metal beaker in an ice-water bath with the small tip

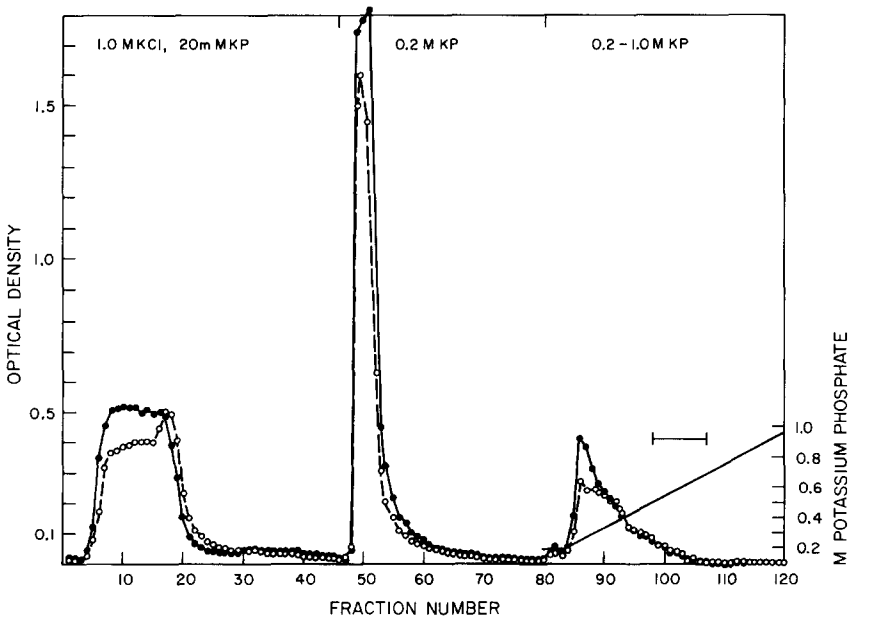

Fig. 1. Chromatography on Hydroxyapatite I. 94 ml of fraction II, containing 5% PEG and 1 M KCl in 20 mM potassium phosphate pH 7.0 and 1 mM β-mercaptoethanol were loaded onto a 8 x 2.5 ml column. The column was washed with 920 ml 1 M KCl in 20 mM potassium phosphate, then with 660 ml 0.2 M potassium phosphate before a linear gradient of 2 x 200 ml potassium phosphate (0.2-1.0 M, pH 7.0) was applied. Fractions containing 20 ml were collected. Distribution of the N-C enzyme is indicated by the bar. OD 260, •; OD 280, o. The gradient was monitored by conductivity measurements.

(0.12") of a Branson Sonifier at step 4. A total sonication time of 2.5 min. was applied in 10 sec. pulses. This lysate (fraction 0) was cleared by centrifugation in a Beckman 30 rotor at 20,000 rpm for 5 hours. The supernatant (fraction I) normally contained traces of floating lipids, which disappeared during the following step.

Precipitation with polyethyleneglycol: Solid PEG, 5% w/v, was dissolved with gentle stirring for 30 min. After 15 to 20 min. a visible precipitate developed which was removed after 30 min. in a Sorvall centrifuge at 10,000 rpm. The supernatant (fraction II) contained 90% of the activity. Most of the chromosomal DNA and approximately 40% of the protein was removed in this step.

Hydroxyapatite I: Fraction II was immediately loaded onto a hydroxyapatite column (2.5 x 8 cm), previously equilibrated with SB containing 1 M KCl. After loading, 23 bed volumes of 1 M KCl in SB, followed by 16 bed volumes of 0.2 M potassium phosphate were passed through the column which retained the enzyme, subsequently eluted between 0.5 and 0.7 M potassium phosphate, in a 400 ml linear gradient of 0.2 to 1.0 M potassium phosphate (Fig. 1) applied at 45 ml/hr. The fractions containing activity were pooled to form fraction III.

DEAE cellulose: Fraction III (110 ml) was diluted to approximately 0.05 M potassium phosphate by addition of 9 volumes of 1 mM MET and loaded onto a previously equilibrated DEAE cellulose column (2 x 6 cm) with a flow rate of ~70 ml/hr. Approximately 96% of the activity passed through unretarded together with 34% of the total protein (fraction IV). The remaining 4% of the activity eluted in a broad peak at about 0.2 M potassium phosphate. Whether the latter activity is identical with the major is not yet known. In a previous procedure (5) all of the activity bound to the DEAE resin. We tentatively attribute the difference to the significant amounts of nuclear DNA present in the earlier preparations.

Hydroxyapatite II: Fraction IV in 1100 ml was immediately loaded onto a

small hydroxyapatite column (1 ml bed volume), previously equilibrated against 0.05 M potassium phosphate. The column retained about 50% of the protein. It was washed with 50 ml 0.05 M, followed by 50 ml 0.3 M potassium phosphate. The enzyme was eluted in a 60 ml linear gradient of 0.3 to 0.7 M potassium phosphate between 0.4 and 0.6 M potassium phosphate (Fig. 2). The protein distribution in the effluent coincided with the N-C activity as monitored on 0.8% agarose slab gels (see legend to Fig. 2). The pooled active fractions (fraction V) contained 0.8 mg protein and 1.85 x 10^6 units. It was obtained from ca. 4 x 10^9 cells. The purification was 58-fold and the overall recovery was 12% (Table 1). The enzyme in 0.5 M potassium phosphate and 1 mM MET appears to be stable over several months when quickly frozen in liquid N_2 and stored at -20°.

Assessment of purity: Fraction V enzyme produced a single protein peak

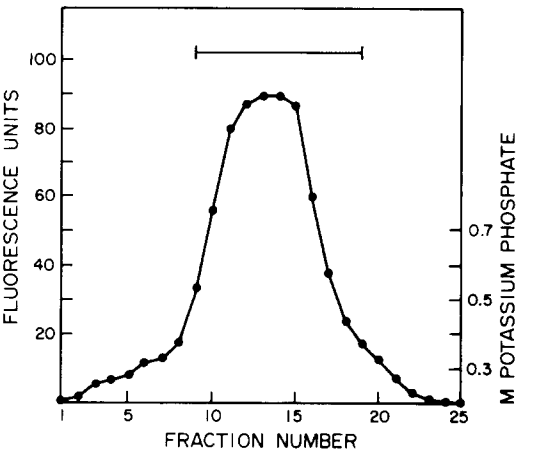

Fig. 2. Chromatography on Hydroxyapatite II. Fraction IV (990 ml) containing 2.52 mg protein was passed over a small hydroxyapatite column (1 ml bed volume, in a 10 ml syringe type B-D Plastipak). The column was first washed with 50 ml 0.05 M potassium phosphate, pH 7, followed by 50 ml of 0.3 M potassium phosphate. The enzyme was eluted in a 60 ml linear gradient of 0.3 to 0.7 M potassium phosphate. The N-C activity was monitored on 0.8% agarose slab gels (Methods) and its distribution is indicated by the bar. The protein distribution was determined by measuring the tryptophane fluorescence at 286 nm excitation wave length and 340 nm emission wave length at room temperature using a Perkin-Elmer fluorescence spectrophotometer, MPF 2A. The machine was used in the ratio mode with sample sensitivity 1 and slits of 8 and 10 for the excitation and emission light respectively.

after elution with a linear potassium phosphate gradient from DNA cellulose, prepared according to Alberts (11). One major protein peak containing the activity was also seen after gel filtration of fraction V enzyme over Sephadex G150. The elution volume corresponded to a molecular weight of ca. 75,000, assuming a globular shape. Electrophoresis of the enzyme (fraction V) on 10% SDS polyacrylamide slab gels (12) gave one band with an apparent molecular weight of ca. 35,000. Chromatography on DNA cellulose and gel filtration did not change the pattern.

2. Demonstration of the enzyme nature: The high specific activity of N-C enzyme made it possible to follow the reaction with DNA in molar excess over N-C enzyme. The protein value was based on Lowry determinations (13) and the assumptions that fraction V contained pure N-C enzyme with a molecular weight of 75,000. The reaction was carried out with PM2 DNA I at 37° in a mixture that contained one unit of N-C enzyme with a specific activity of 1.85×10^6 units/mg per μg of PM2 DNA (MW 6×10^6). The reaction (a 20-fold excess of DNA over N-C protein molecules) was followed by analyzing paired samples fluorometrically and by buoyant density centrifugation in EtdBr-CsCl.

PURIFICATION OF NICKING-CLOSING ENZYME*

	Fraction	Vol. (ml)	Total Protein (mg)	Total Activity (units)	Spec. Activity (units/mg)	Purification
0	Sonicated nuclei	100	370	15×10^6	4×10^4	x 1
I	Supernatant of sonicated nuclei	98	277	14.7×10^6	5.3×10^4	x 1.3
II	PEG	94	183	12.5×10^6	6.9×10^4	x 1.7
III	Hydroxyapatite I	98.5	5.14	7.2×10^6	1.4×10^6	x 40
IV	DEAE cellulose	990	2.52	4.8×10^6	1.9×10^6	x 47.5
V	Hydroxyapatite II	31.8	0.80	1.85×10^6	2.31×10^6	x 58

*Prepared from 4×10^9 cells.

Table 1. The activity in fraction 0 was determined in the buoyant separation method. The activity in fraction I-V was determined by the fluorometric method. Protein concentrations were measured according to the Lowry procedure (13).

Fifty percent of the maximal change in the fluorescence intensity was observed after 7 minutes. The reaction was complete after ca. 45 min., a result confirmed by centrifugation. We conclude that a N-C protein molecule acts repeatedly and is able under the conditions of this experiment to relax at least 20 DNA molecules completely within 45 min.

The time required for 50% reaction was 7 min. instead of 30 min. as expected for 1 unit of enzyme, and the initial reaction rate was 4-fold greater than observed earlier in the standard reaction volume (experiment not shown). The principal difference was that the reaction volume was 3.5 ml instead of 0.25 ml with attendant smaller losses to walls of the reaction vessels and pipettes and/or denaturation at the water-air interface.

DISCUSSION

The present procedure incorporates three major improvements over the

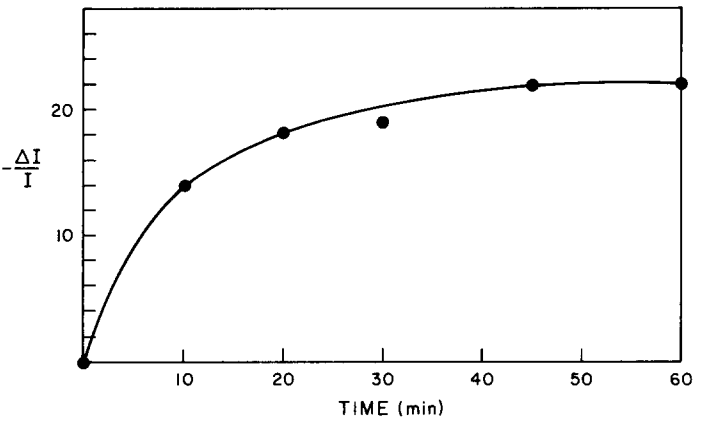

Fig. 3. Demonstration of the Enzymatic Character of the N-C Activity. PM2 DNA I was incubated after mixing the components at 0° with an enzyme to DNA ratio of 1 unit of N-C enzyme to 1 µg of DNA as described in the text. DNA was in approximately 20-fold molar excess over the enzyme as calculated from the specific activity of the N-C enzyme preparation used in this experiment (1.85×10^6 units/mg). The reaction was followed by removing 250 µl samples containing 1 µg of DNA after various times diluting into 2.75 ml of an ethidium bromide containing buffer solution (0.5 µg/ml EtdBr in 10 mM Tris pH 7.6 and 0.1 mM EDTA). The fluorescence emission was observed at 584 nm with an excitation wave length of 365 nm as described earlier. I is the fluorescence intensity measured after 0 minutes (in arbitrary units). ΔI is the difference between the fluorescence intensities of samples withdrawn after various times and the initial intensity I. The reaction was complete after 45 min. as indicated by the absence of any further decrease in the fluorescence intensity.

previous one (5): apparently complete solubilization of the enzyme upon sonication of the nuclei in high salt, removal of the nuclear DNA in the early purification steps with losses of less than 50%, and the high and reproducible overall yields of 12% under the assumption that crude extracts do not contain stimulating factors. The 0.8 mg recovered as N-C enzyme represent ca. 1 to 2% of the total nuclear protein. This large value accounts for the small (58-fold) increase in specific activity (Table 1) even though the protein was extensively purified.

The relaxation process requires a temporary chain break in order to allow the rotation of one strand around the intact opposite strand. Our present data do not distinguish whether the reaction under standard conditions (see Materials and Methods) proceeds stepwise, releasing one superhelical turn at a time and then closing (6) or whether all the turns are removed after the first hydrolytic event. With one unit of enzyme we observe an all or none process and never see a gradual shift of the whole DNA population to the position of completely relaxed DNA in the centrifugation or gel analyses. We, therefore, conclude that the enzyme has a preference for completely relaxing one DNA molecule before it reacts with another one.

With respect to its role *in vivo* the N-C enzyme is likely to be a factor promoting replication by providing a swivel ahead of the replication fork. The finding that the enzyme appears to be predominantly associated with the condensed, template inactive regions (Vosberg and Gottesfeld, unpublished) in isolated chromatin from rat liver underlines its involvement in replication. Alternatively, the N-C enzyme could be involved in transcription by promoting nascent RNA chains through transient swivels in the DNA ahead of the growing point.

Recently published evidence (14,15) suggests that changes in the superhelix density in closed circular SV40 DNA occur upon removal of the histones from SV40 "mini chromosomes" and "nucleosomes". These results indicate that the free DNA in the DNA-histone complex is relaxed perhaps as a result of

the N-C enzyme action and suggests that the structure and/or organization of the histone bound DNA is responsible for the formation of the superhelical turns upon DNA purification.

Finally, work in progress in this laboratory by D. E. Pulleyblank and the present authors has shown that the main polypeptide present in highly purified enzymes from mouse LA9 cells, rat liver, and calf thymus comigrate under a variety of conditions with a subfraction of the lysine rich histone H1 prepared from the homologous sources. These results indicate either that the N-C enzymes from these sources copurify with the H1 histone, or that the activity is a property of one or more subfractions of histone H1.

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