A DNA NICKING-CLOSING ENZYME FROM MOUSE L CELLS

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ABSTRACT

A DNA nicking-closing activity was isolated from mouse LA9 cells. This activity converted native closed circular DNA into a limit product set of topological isomers having a mean degree of supercoiling of ~ 0. These isomers differed from each other by single superhelical turns, and their relative masses fit a Boltzmann distribution. By re-reacting each isomer with the activity, the original distribution was regenerated. Therefore supercoiling of the DNA substrate was not required for the action of the enzyme. Polynucleotide ligase, acting on nicked circular DNA, formed under the same conditions, the same distribution of topological isomers. Therefore the nicking-closing enzyme must have generated nicked intermediates in which rotations at the nick were constantly occurring as a result of the thermal fluctuation of the DNA polymer chain. During resealing of the DNA, the enzyme froze the intermediates into a set of isomers differing by integral number of turns.

Initial purification of the mouse enzyme showed copurification of the activity with histone H1. It was not clear whether H1 was the enzyme or a trivial contamination. In Chapter 2, I present a purification of the nicking-closing enzyme identifying it as a monopolypeptide of molecular weight 68,000. I show that the mouse enzyme is not similar to histone H1, that it is a rather acidic protein, and that its molecular weight is similar to that of the rat liver and the human KB cell enzymes.

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Biochemistry

Action of nicking-closing enzyme on supercoiled and nonsupercoiled closed circular DNA: Formation of a Boltzmann distribution of topological isomers

(polynucleotide ligase/gel electrophoresis)

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Contributed by Jerome Vinograd, August 25, 1975

ABSTRACT **Highly purified nicking-closing enzyme from mouse cells in 20-fold enzyme/substrate excess converts closed circular native PM2, ColE1, and Minicol DNA into limit product sets of DNAs. Each set has a mean degree of supercoiling of approximately zero. The individual species in the sets differ by $\Delta\tau = \pm 1, \pm 2$, etc., and the relative masses fit a Boltzmann distribution. It was also demonstrated that "nonsupercoiled" closed circular duplex molecules serve as substrates for the nicking-closing enzyme, and that a distribution of topological isomers is generated. Polynucleotide ligase, acting on nicked circular DNA, forms under the same conditions, the same set of closed DNAs. The latter enzyme freezes the population into sets of molecules otherwise in configurational equilibrium in solution.**

Nicking-closing (N-C) activities that alter the topological winding number (α) of closed circular DNA occur widely in nature (1–5). The topological winding number is the number of revolutions that one strand makes about the other if the molecule is constrained to lie in a plane. The activities have been demonstrated to be enzymatic with proteins from *Escherichia coli* (6), mouse (7), and human (8) cells in culture. The N-C enzyme from mouse LA9 cell nuclei, purified to homogeneity in good yield, is a major constituent of chromatin and accounts for about 1% of the total protein (H-P. Vosberg and J. Vinograd, unpublished work). It is similar to other eukaryotic N-C enzymes in its ability to relax both positive and negative superhelical turns. A probable *in vivo* role for the enzyme is to provide the transient swivels required for DNA replication. Such swivels may also be required in transcription, and in the condensation and decondensation of chromatin.

In this study we have examined the limit product of the action of N-C enzyme on several closed circular DNAs by gel electrophoresis. Under appropriate analytical conditions, the limit product separates into a set of species differing in topological winding number. Individual species, isolated from the set, regenerate the original distribution upon incubation with the N-C enzyme. We view the foregoing as the consequence of four necessary events: nicking of the DNA, relaxation, random rotation about the swivel, and closure (Fig. 1). A set of species is also found when *E. coli* polynucleotide ligase is used to close a nicked circular DNA (9, 10). It is shown here that distribution of products obtained with ligase is indistinguishable from that obtained with N-C enzyme when the incubation conditions are the same for both reactions.

The relative masses of the species, when plotted against α, fall on a Gaussian curve. Such a curve is anticipated for a Boltzmann distribution, when the energy of supercoiling is proportional to the square (11) of the degree of supercoiling. The similarity of the products obtained with two different enzyme systems and the further similarities of the values of the free energy of supercoiling obtained here and by nonenzymatic procedures strongly indicate that the Boltzmann distributions are characteristic of the thermally induced torsional fluctuations of free DNA uninfluenced by the presence of an enzyme.

Abbreviations: N-C, nicking-closing; EtdBr, ethidium bromide.

MATERIALS AND METHODS

Enzymes and DNA. *E. coli* polynucleotide ligase was a gift of Dr. H. Boyer. N-C enzyme was prepared from mouse LA9 cell nuclei (H-P. Vosberg and J. Vinograd, unpublished work). DNase I was purchased from Worthington. PM2 DNA was prepared according to (12). ColE1 DNA was prepared from bacterial strain JC411 (ColE1) (13). Minicol DNA was prepared from bacterial strain PVH51 supplied by Dr. H. Boyer (14).

Relaxation of Closed Circular DNA. DNA (5–50 μg/ml) was incubated in 0.2 M NaCl, 0.01 M Tris·HCl, 0.1 mM EDTA at pH 7.4 with 5–20 units of N-C enzyme per μg of DNA for 24 hr at 37°. When relaxations were performed at other temperatures, a second addition of 10 units of enzyme per μg of DNA was made after 24 hr and the incubation was continued for a further 24 hr. Reactions were terminated by the addition of sodium dodecyl sulfate to a final concentration of 0.1%. For comparison of the enzymatically relaxed DNA with ligase closed DNA, both reactions were carried out in 0.2 M NaCl, 3 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 33 μM NAD, 0.2 mM EDTA, 0.1 mM spermidine, 20 μg/ml bovine

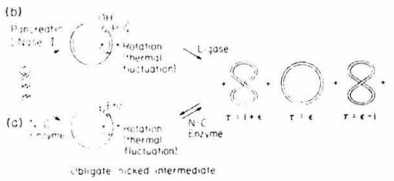

FIG. 1. The formation of a Boltzmann distribution of topological isomers of closed circular DNA. (*a*) The action of N-C enzyme on a closed circular DNA substrate. The diagram is not intended to specify the mechanism for N-C action. The obligate elementary steps include nicking, releasing preexisting supercoils, random rotation at the swivel, and closure. The quantity ϵ is the difference between the duplex winding number (β) of a nicked DNA and the duplex winding number of a similar hypothetical molecule with $\tau = 0$. The fractional turn ϵ is not illustrated in Fig. 1. (*b*) The closure of nicked circular DNA by polynucleotide ligase.

serum albumin, 30 mM Tris·HCl at pH 7.8 for 8 hr at 37°. Five micrograms of DNA and 140 units of N-C enzyme and/or 2×10^{-3} units of ligase were used in the reactions.

Electrophoresis. A vertical slab gel electrophoresis apparatus (Aquebogue) was used. Gels contained 1% agarose (Sea Kem), 40 mM Tris·acetate at pH 7.8, 0.5 mM EDTA. Magnesium acetate (5 mM) was added in most instances. Samples (5–100 μl) containing 200–400 ng of DNA were layered into the sample wells in a solution containing approximately 10% Ficoll 70 (Pharmacia) and 2 mM EDTA at pH 8. Up to 250 μg of DNA in 1 ml of the same solution was layered into the large sample well of a preparative gel. Two volts per cm were applied to the 8 mm preparative gels and 3 V/cm to the 4 mm analytical gels. Electrophoresis times were varied according to voltage gradient, molecular weight of the DNA, and temperature.

Determination of Relative Masses of DNA Species by Fluorescence Photography. Gels were stained in the dark overnight in 10 mM Tris·HCl, 2 mM EDTA, 2 μg/ml of ethidium bromide (EtdBr). The gels were illuminated from below with short wavelength ultraviolet light from a Transilluminator (Ultra Violet Products Inc.) and photographed on Kodak Plus X film. The films were traced on a Joyce-Loebl microdensitometer. Traces were evaluated with a Hewlett Packard 9864A Digitizer Platen and 9820A calculator. Relative fluorescence intensities (J_i) were evaluated and integrated over the band to obtain the relative mass with the equation

$$\sum_h J_i \Delta x_i = \sum_h (10^{D_i/\gamma} - 1)\Delta x_i \qquad [1]$$

where D_i is the optical density above background and γ is the slope of the characteristic curve of the film (γ was evaluated for each film). Where appropriate, the areas under each peak in the optical density traces were also evaluated. The areas give a good approximation to the relative concentrations of species providing $D/\gamma \ll 1$. Procedures for calculating ϵ and B (defined below) for the distributions were incorporated into the integration program. A full description of the experimental method will be presented elsewhere (D. E. Pulleyblank and J. Vinograd, unpublished work).

Extraction of DNA from Gels. Stained gels were placed on a mask with 3 mm slots parallel to the direction of the electrophoresis, and portions of the DNA bands were visualized by illumination from below. The gel was sliced so as to separate bands, and the sections that had received direct UV illumination were discarded. The remaining gel fragments were frozen and thawed three times and then centrifuged at 40,000 × g for 1 hr. The supernatant, containing approximately 30–50% of the DNA in the gel slice, was freed of ethidium by extraction with 1-butanol. The DNA was precipitated from the aqueous phase by the addition of 2 volumes of cold ethanol, followed by centrifugation in an SW 50.1 rotor at 35,000 rpm at 4° for 1 hr.

RESULTS

The Existence of Multiple Species of Closed Circular DNA in the Limit Product of N-C Enzyme Action. Keller and Wendel (4) were able to resolve a series of species in partially relaxed closed circular simian virus 40 DNA using agarose gel electrophoresis. Their assumption that adjacent, resolved species differ by a single superhelical turn has been used throughout the present study of DNAs with low numbers of superhelical turns. The validity of the assumption is considered in the *Discussion* section.

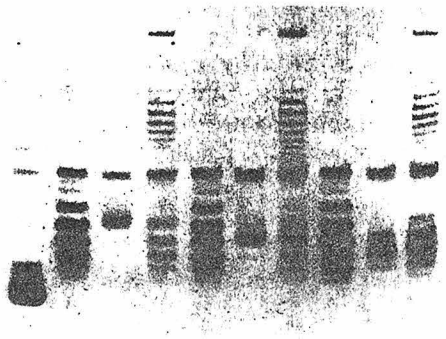

FIG. 2. Demonstration that non-supercoiled DNA is a substrate for the action of N-C enzyme and that N-C enzyme reacts with a homogeneously supercoiled species of closed circular DNA ($\tau \approx 0$) to form a thermal distribution of species. (*1*) Minicol I and II; (*2, 5,* and *8*) limit products of the initial reaction of Minicol I with N-C enzyme; (*3, 6,* and *9*) purified species with $\tau \approx +1, 0, -1$, respectively; (*4, 7,* and *10*) limit products regenerated after reaction of purified species with N-C enzyme. Bands migrating slower than Minicol II in channels 4, 7, and 10 are sets of relaxed ColE1 DNA. Native ColE1 DNA was added to the reaction mixtures to monitor the activity of the enzyme.

In a study of the effects of electrophoresis conditions on the resolution of closed circular DNAs with differing values of α, it was found that the limit product of N-C enzyme action on closed circular DNA could be resolved into multiple species. Optimum resolution was achieved at 4° in the presence of 5 mM Mg^{++}. Under these electrophoresis conditions, the mean duplex winding number (β) is greater than during the relaxation reaction (15). The increase is compensated by the generation of $\Delta\tau$ negative superhelical turns in all members of the set according to the equation $\Delta\beta = -\Delta\tau$. Comigration of pairs of species that initially had equal numbers of positive and negative superhelical turns is eliminated, and the magnitudes of τ are such that the gel resolves species that differ by unit values of τ.

The Generation of a Set of Species from an Isolated Homogeneous Species. The thermal nature of the distribution observed in the limit products of the N-C enzyme action on closed circular DNA has been established with experiments such as shown in Fig. 2. The three dominant species of Minicol DNA present in the N-C enzyme limit products (channels 2, 5, and 8) were isolated separately from a preparative agarose gel and purified. These materials (channels 3, 6, and 9) were treated with the N-C enzyme under the conditions used to generate the original set of limit products. In each case a new set of products was generated (channels 4, 7, and 10) with the same distribution as the original set.

The above results lead to the following conclusions. (*i*) The species found in the original reaction mixture are the limit products of the reaction and are not generated by incomplete relaxation of the supercoiled substrate. Therefore, under the conditions of reaction, the materials in channels 3, 6, and 9 contained approximately +1, 0, and −1 superhelical turns, respectively. (*ii*) Species are generated with higher and lower superhelical winding numbers than the substrates for the rereaction. Rotation about the swivel must, therefore, be driven by thermal fluctuations. The creation of a multi-

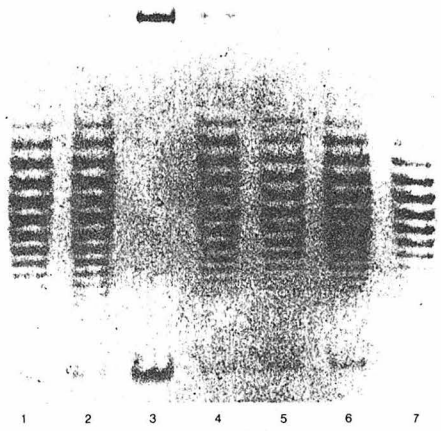

FIG. 3. The limit product of the N-C enzyme is indistinguishable from the ligase-closed product. (*1*) PM2 II + Ligase; (2) PM2 I + N-C Enzyme; (*3*) I, II; (*4*) mixture of products in (*1*) and (2); (5) PM2 I, II + N-C Enzyme+Ligase; (*6*) PM2 II + N-C Enzyme+Ligase; (7) ligase closed PM2 + N-C Enzyme. Reactions were carried out in 0.2 M NaCl, 3 mM $MgCl_2$, 5 mM $(NH_4)_2SO_4$, 33 μM NAD, 0.2 mM EDTA, 0.1 mM spermidine, 20 μg/ml of bovine serum albumin, 30 mM Tris-HCl at pH 7.8 for 8 hr at 37°; 5 μg of DNA, 140 units of N-C Enzyme, 2×10^{-3} units of ligase were used in the reactions. Nicked and linear DNA were removed by EtdBr–CsCl buoyant centrifugation. PM2 I (10 ng) was added to each channel.

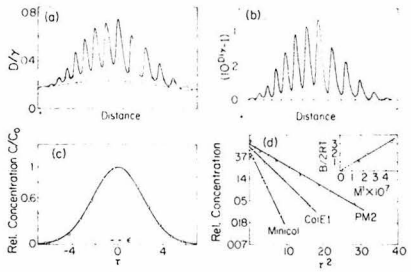

FIG. 4. Processing of data for the relative masses in each set. The Boltzmann distribution. (*a*) Absorbance trace of a photograph of an ethidium stained gel of relaxed PM2 DNA with background traces from both sides of the sample channel. Sample background (- - -) was calculated by averaging the background traces. The ordinate is plotted in units of D/γ. (*b*) Plot of fluorescence intensity against distance, generated from the optical density trace shown in 4a. The curve was calculated by the equation $J = 10^{D/\gamma} - 1$, where D is the optical density above background. The peaks were integrated between the indicated limits, to determine the relative masses of the species present. (*c*) The concentration of species present in solution are normalized by C_0, the concentration of a theoretical species lacking supercoils. The curve through the points is the calculated Gaussian curve with the best least squares parameters, 0.105 and 0.33 for $B/2RT$ and ϵ, respectively. The point lying above the curve at $\tau = 0.67$ was not used in the least squares fit because of contamination of this species by linear PM2 DNA. (*d*) The natural logarithm of the relative masses of species in the limit products of three closed DNAs treated with N-C enzyme, plotted against the square of the superhelical winding number. The intercepts of the traces have been displaced for clarity of presentation.

ple set from a single species represents an increase in the entropy of the system. (*iii*) Supercoiling of the closed circular substrate is *not* a requirement for the action of the mouse N-C enzyme. Linear and nicked circular DNAs can therefore, with confidence, be considered substrates for the enzyme.

Sets of Species Formed with N-C Enzyme and Closed Circular DNA Are Indistinguishable from Those Formed with Ligase and Nicked Circular DNA. Ligase closure of nicked circular DNA also leads to a set of species with different topological winding numbers (10). The distribution of species was compared with the distribution of N-C enzyme products (Fig. 3). Since environmental conditions affect the duplex winding number, and hence the position of the center of the distribution (see below), it was necessary to perform the reactions under conditions that were as similar as possible. The observed distributions were indistinguishable (channels 1 and 2). The addition of a second enzyme and/or substrate to a reaction mixture caused no detectable change in the distributions (channels 4, 5, 6, and 7), and ruled out the possibility that the correspondence between the distributions in channels 1 and 2 was due to adventitious effects of protein binding. The N-C enzyme did not close the ligase substrate PM2 II (data not shown).

We conclude that the forms of the distributions generated by each of the two systems are the same, with respect to both the center of the distribution and, with less accuracy, to the relative concentrations of the species present. The comparison of products generated by the two enzymatic systems corroborate conclusion (*i*) of the previous section.

The Boltzmann Distribution. A set of molecules at thermal equilibrium contains a distribution of states with respect to all degrees of freedom available to the molecules. The number of molecules in a given state (N_i) within the set (N_t) is related to the energy of the state (E_i) by the Boltzmann equation

$$N_i/N_t = A \exp(-E_i/RT) \quad [2]$$

where A is a normalization factor; R and T have their usual meanings. A closed circular DNA with a given value of α cannot come to thermal equilibrium with respect to α, because of the requirement for breakage and reformation of a covalent bond in the phosphodiester backbone. The N-C enzyme catalyzes these reactions, and allows the system to come to equilibrium.

The molar free energy associated with supercoiling of closed circular DNA (G_τ) has been determined for superhelical simian virus 40 DNA (11) and PM2 DNA (15) and is related to the number of superhelical turns by the equation

$$G_\tau = (B\tau^2/2) \quad [3]$$

where B is a constant. The superhelix density, $\sigma = 20\,\tau/N$ where N is the number of nucleotides in the DNA is substituted into [3]

$$G_\tau = B(N\sigma/20)^2/2 \quad [4]$$

We observe, providing G_τ is proportional to N when σ is constant, that B must be inversely proportional to N.

Upon incorporating Eq. [3], the Boltzmann Eq. [2] becomes

$$N_i/N_t = A \exp(-B\tau^2/2RT) \quad [5]$$

The equation is Gaussian, and a plot of ln N_i against τ^2 should be linear. We have defined ϵ ($-0.5 < \epsilon \leq 0.5$) as the difference between the duplex winding number β of a

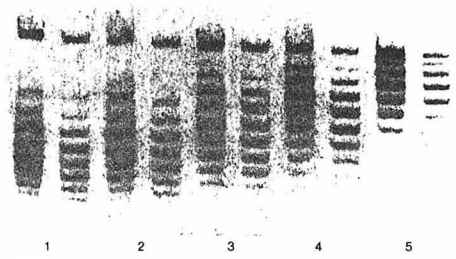

FIG. 5. The effect of the incubation temperature on the position of limit products in a slab-gel electrophoresis experiment. The paired samples contained 100 and 200 ng of DNA. The incubation temperatures were 41.5°, 37.2°, 29.6°, 22.5°, and 13.4° in (*1*) to (*5*).

nicked DNA and the duplex winding number of a similar hypothetical closed molecule with $\tau = 0$ in the same environment. The number of superhelical turns in a member of the distribution is defined by the equation $\tau = I + \epsilon$ where I is an integer.

The relative concentrations of the species present in the limit products of N-C enzyme action on Minicol, ColE1, and PM2 DNAs were determined from traces of photographs of ethidium stained gels (Fig. 4a and b). The relative concentrations were fitted by a least squares procedure to a Gaussian equation to determine the best value for ϵ and $B/2RT$ (Fig. 4c). The natural logarithm of the relative concentrations are plotted against τ^2 (Fig. 4d). The slopes of the lines ($B/2RT$) are inversely proportional to the molecular weight of the DNA (Fig. 4d, insert); this is in agreement with the prediction made above. The values of $B/2RT$ measured at 37° for PM2, ColE1, and Minicol DNA were 0.10, 0.17, and 0.32, respectively. The reproducibility was ±10% for measurement of 16 channels in two gels for ColE1 DNA, and measurements of 12 channels in two gels for PM2 and Minicol DNAs. Note that the number of species observed is approximately proportional to the square root of the molecular weight of the DNA (Fig. 4d).

The Thermal Unwinding of DNA. The value of the equilibrium duplex winding number (β) is sensitive to small changes in the environment of the DNA. Analysis of the distributions obtained after N-C enzyme action on closed circular DNA give accurate values for ϵ. Changes in β can be measured by counting the number of turns including partial turns (ϵ) between the center of a sample distribution and the center of a reference distribution. In general ϵ can be estimated to within ±0.1 duplex turn; this corresponds to a limit of accuracy for PM2 DNA of about ±0.2 turn in the 10^3 duplex turns.

Temperature and protein binding are examples of factors that affect β and $\bar{\alpha}$ (see *Discussion* for the definition of $\bar{\alpha}$). Here closed circular PM2, ColE1, and Minicol DNA samples were relaxed at different temperatures (Fig. 5). Each distribution was analyzed by the least squares procedure to obtain the value of ϵ, and hence the position of the center of the distribution. One of the species in the distributions was used as an arbitrary marker, and the number of helical turns to the center of each distribution was calculated and plotted against the temperature of reaction (Fig. 6). The temperature dependence of the rotation angle was $-1.4 \pm 0.1 \times 10^{-2}$ °/C°, base pair.

Quantitation of the mass distributions in Fig. 5 showed that the values of $B/2RT$ were constant to within ±10%,

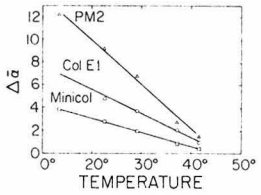

FIG. 6. The temperature dependence of the center of the Boltzmann distribution. Each distribution was analyzed by the least squares procedure to obtain the value of ϵ, and hence the position of the center of the distribution. A corresponding band was used in each set as a reference; the number of turns including partial turns (ϵ) to the center of each distribution was measured. The center of the distribution corresponds to $\bar{\alpha}$ as defined in the *Discussion*.

which indicates that the entropy of supercoiling is the major component of the superhelix free energy.

DISCUSSION

The assumption that each resolved species differs from its neighbors by a single turn in its topological winding number is supported by two arguments. It is the simplest explanation for the observation of discrete bands. Other proposals, for example, that adjacent species differ by two turns in the topological winding number, appear artificial and especially unlikely in view of the similarity of the results obtained here with N-C enzyme and polynucleotide ligase. If a shift in the center of the distribution by a single band is equivalent to a 360° change in the winding of the duplex, we calculate the thermal unwinding angle of duplex DNA to be $-1.4 \pm 0.1 \times 10^{-2}$ °/C°, base pair. This value corresponds well with $-1 \pm 0.2 \times 10^{-2}$ °/C°, base pair calculated from the results of Wang (16) by using 26° instead of 12° for the unwinding angle (ϕ) of ethidium when bound to DNA (17, 18).

The Free Energy of Supercoiling. The free energy of supercoiling has been determined in the past by studying the relative binding affinities of a closed circular DNA and its nicked circular counterpart for the unwinding ligand, ethidium (11, 15). To compare the more direct results presented here with those obtained previously, it is necessary to know ϕ. Provided that the DNA is negatively supercoiled, a term ν_c is defined as the molar ratio of bound ethidium to DNA phosphate when all superhelical turns have been removed. If ϕ is expressed in degrees $\tau = N\phi\nu_c/360$, where N is the number of nucleotides in the DNA. Eq. [3] can then be written

$$G_t = (B/2)(N\phi\nu_c/360)^2 \qquad [6]$$

As noted previously, B is inversely proportional to the molecular weight of the DNA. We define a molecular weight independent term $b \equiv BN/2$ to compare the present results with those obtained previously by others. Eq. [3] assumes the form $G_\tau = b\tau^2/N$ where G is in cal mol^{-1} when RT is in cal mol^{-1}. The value of b/RT calculated for the three DNAs in the present study is $2.06 \pm 0.14 \times 10^3$. We have reevaluated the coefficient of Eq. [**24**] of Bauer and Vinograd (11), with the more recent value of 26° for ϕ, and obtained 1.78×10^3. With the value of a_1 as defined and determined by Wang (ref. 15, cf. Eq. [**14e**] and Eq. [6] above), we obtain a value for b/RT of 1.05×10^3. The correspondence between these values is satisfying in view of the widely differing experimental methods and the present uncertainties about the cor-

rect value of ϕ (18). It should be pointed out that the correspondences may be the result of compensating errors, in particular since the salt concentration was 0.2 M instead of 3.0 and 5 M in the previous studies.

Superhelix Density Heterogeneity and the Relationships among the Winding Numbers. We define a time dependent variable β_t as the number of duplex turns in a nicked circular DNA molecule. The equilibrium duplex winding number β is defined as the time-averaged or ensemble-averaged value of β_t. Since the system of closed circular molecules generated by the closure of a nicked circle is not homogeneous with respect to α, we define a new term $\bar{\alpha}$ as the median of the Gaussian curve that fits the Boltzmann distribution in α (Fig. 4c). The term $\bar{\alpha}$ is equal to the value of β at the time of ring closure, and approximates the average value of α. In addition, the term $\bar{\tau}$ is defined by $\bar{\tau} = \bar{\alpha} - \beta$. Under the conditions of ring closure, $\bar{\tau} = 0$, whereas individual molecules have values of $\tau = I + \epsilon$, where I is integral. The equation $\bar{\tau} = \bar{\alpha} - \beta$ replaces, in the case of a Gaussian distribution, the previous equation, $\tau = \alpha - \beta$, derived for a single species.

Mechanism of Action of N-C Enzyme. The results presented here do not allow us to distinguish between alternatives for one aspect of the N-C enzyme action on "nonsupercoiled" DNA: the single turn mechanism in which rotation at the swivel is limited to one turn during a nicking-closing event, and the multiturn mechanism in which several rotations can occur during the nicking-closing event. The direction of rotation in both cases would be biased by the free energy of supercoiling. Comparable alternative explanations have been considered for the appearance of intermediates during the relaxation of highly supercoiled DNA (4, 7).

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PURIFICATION OF A DNA NICKING-CLOSING ENZYME FROM MOUSE L CELLS*

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Running Title: Mouse cell nicking-closing enzyme

SUMMARY

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.

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.

EXPERIMENTAL PROCEDURE

Materials

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 6 000 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.

Methods

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 5×10^{5} cells/ml.

Nicking-Closing Enzyme Assay. The assay, which is similar to that previously described (6,11), is based on the increased uptake of ethidium bromide by closed circular DNA when the number of superhelical turns is decreased by enzyme activity. There is a corresponding change in the

electrophoretic mobility of the DNA. The reaction mixture (100 µl) contained 0.2 M NaCl, 0.02 M potassium phosphate (pH 7.3), 2 x 10^{-4} M EDTA, 100 µg/ml BSA, 1 µg of PM2 DNA I, 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° for 30 min after which the reaction was stopped by adding SDS to 0.1% and chilling to 0°. 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). One unit of activity is defined as the amount of enzyme converting half of 1.0 µg of PM2 DNA I to I_o in 30 min.

Spin Dialysis. Samples were desalted by centrifuging through Sephadex G-25 as in Neal and Fiorini (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°, the pH was determined also at 4°.

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°. 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.

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° for 22 hours in a SW50.1 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 cellular 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 centrifugation at 12,100 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. 2). 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 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 (pH 7.3), 0.1 mM PMSF (Fig. 2). Fractions of 6.5 ml were collected. The column was calibrated 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 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.

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 2.8×10^4 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 "Experimental Procedures." 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 "Experimental Procedures"), 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 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 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 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 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 ± 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 polymerase a pI of 5.3 (24), and HeLa RNA polymerase II a pI of 4.7 (25).

The specific activity of fraction V is 2.74×10^8 u/mg. Assuming that the proteins eluting at the active peak have an average molecular weight of 70,000, this specific activity indicates that over a thousand PM2 DNA I molecules may be relaxed to I_o by one protein molecule. Whether the

enzyme achieves this 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.

Acknowledgements

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Footnotes

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.

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.

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

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.

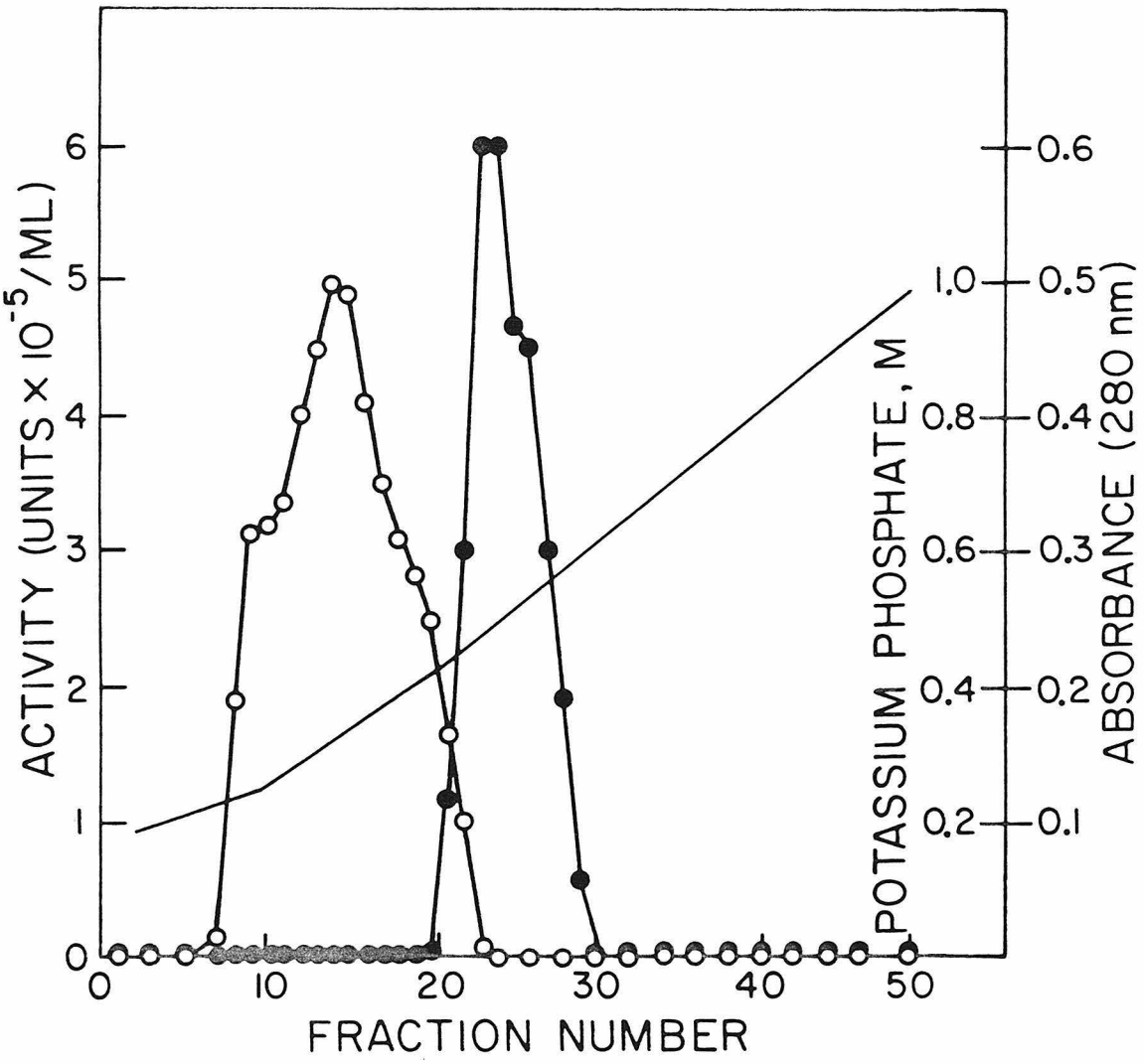

ACTIVITY (UNITS × 10⁻⁵/ML)
POTASSIUM PHOSPHATE, M
ABSORBANCE (280 nm)
FRACTION NUMBER
0
1
2
3
4
5
6
0.2
0.4
0.6
0.8
1.0
0.1
0.2
0.3
0.4
0.5
0.6
0
10
20
30
40
50

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.

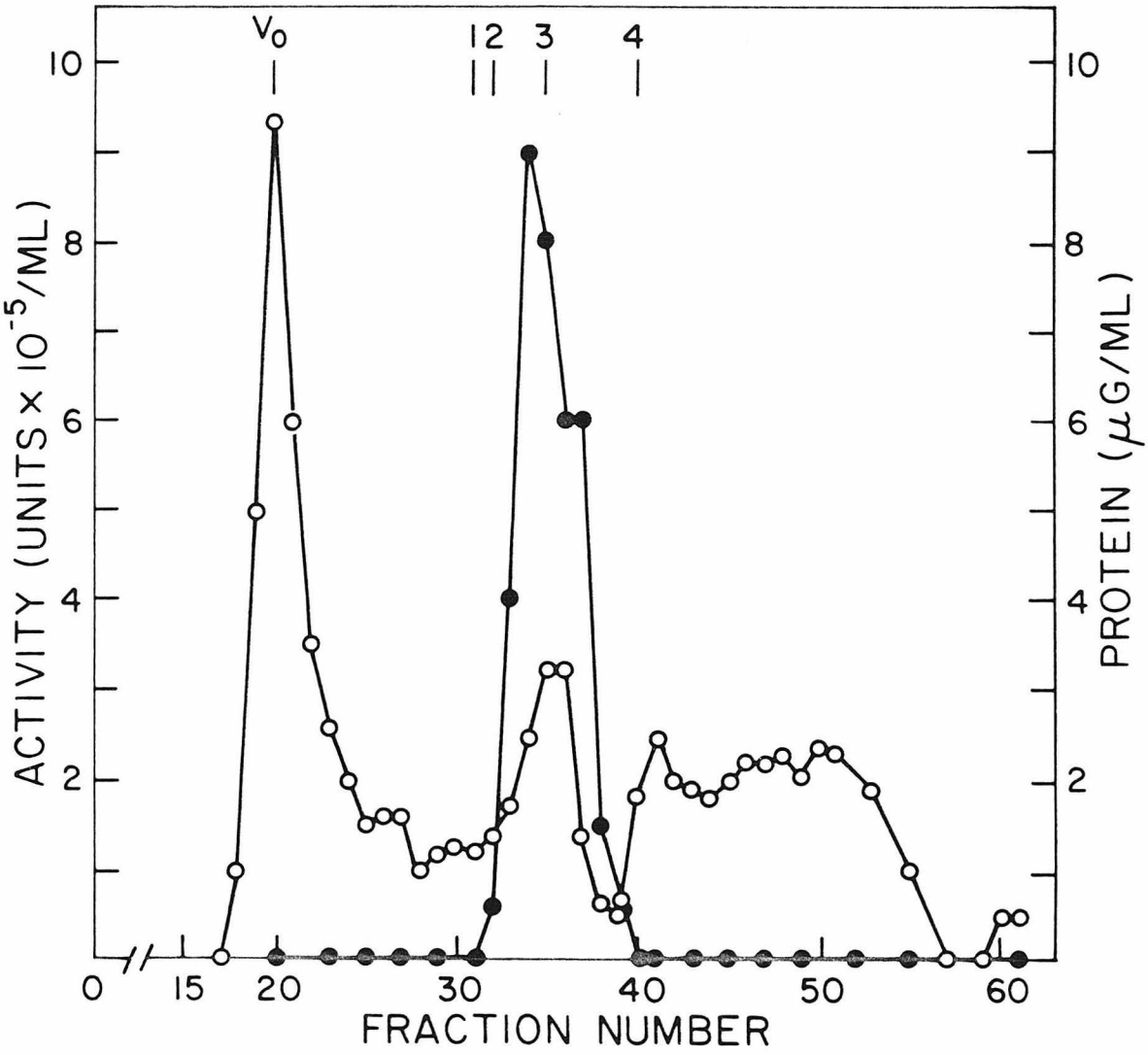

V_0
12
3
4
ACTIVITY (UNITS × 10^{-5}/ML)
PROTEIN (μG/ML)
FRACTION NUMBER
0
15
20
30
40
50
60
2
4
6
8
10

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 "Experimental Procedures"). After 13 hours focusing, 50 µl fractions were collected at 4°, and assayed immediately for DNA nicking-closing activity.

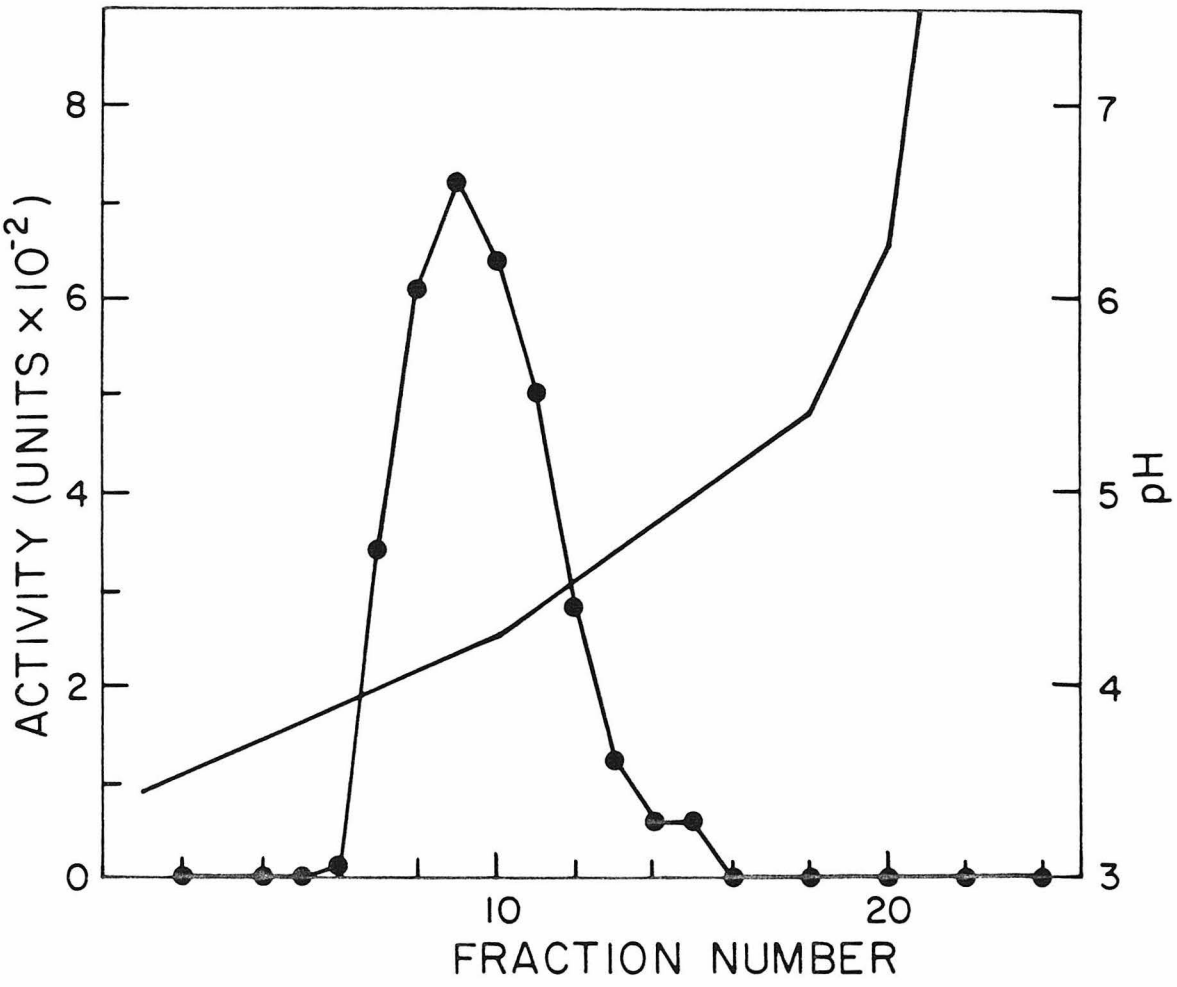

ACTIVITY (UNITS x 10⁻²)
0
2
4
6
8
pH
3
4
5
6
7
10
20
FRACTION NUMBER

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 "Experimental Procedures." 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 mixed with the same set of unlabeled proteins and electrophoresed. The slab gel was Coomassie 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.

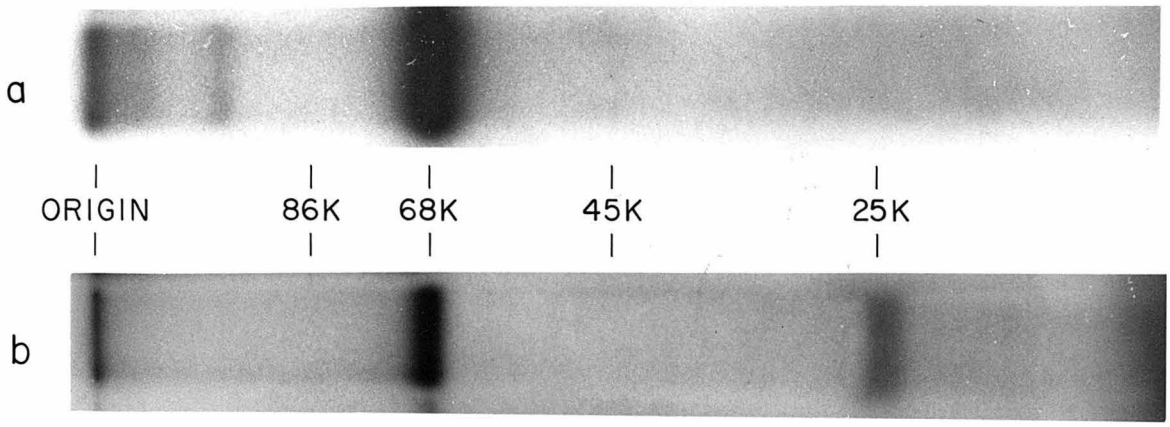

a
ORIGIN
86K
68K
45K
25K
b

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).

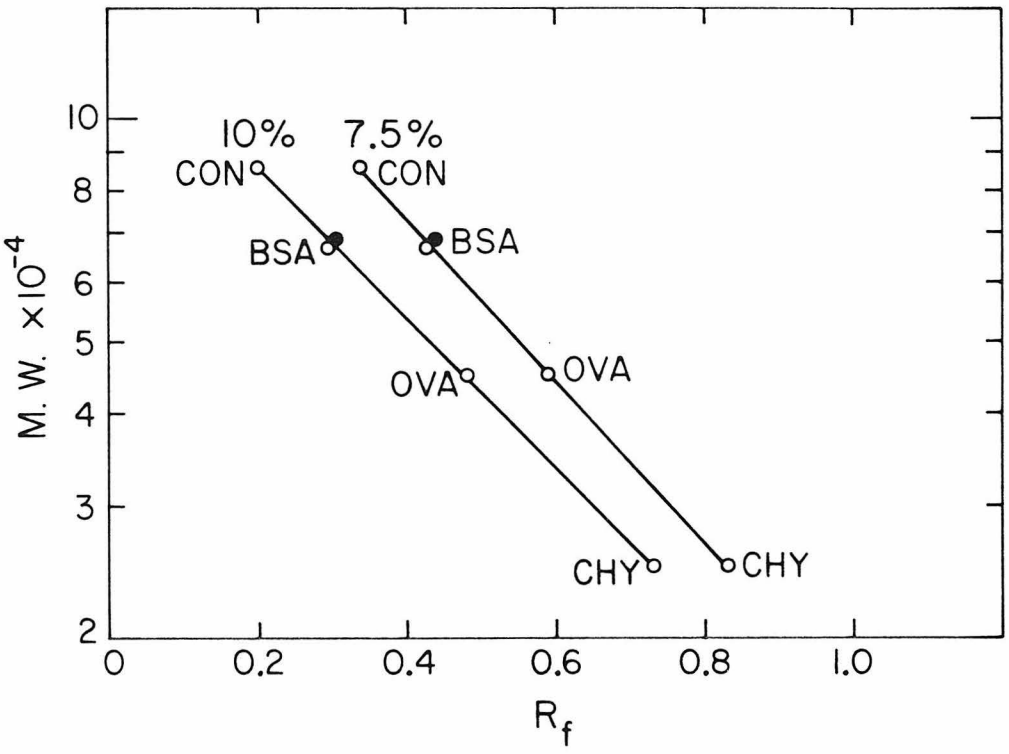

10%
CON
7.5%
CON
BSA
BSA
OVA
OVA
CHY
CHY
M. W. x10⁻⁴
10
8
6
5
4
3
2
0
0.2
0.4
0.6
0.8
1.0
Rf

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 phasminogen, BSA (4.4s), and ovalbumin (3.7s).

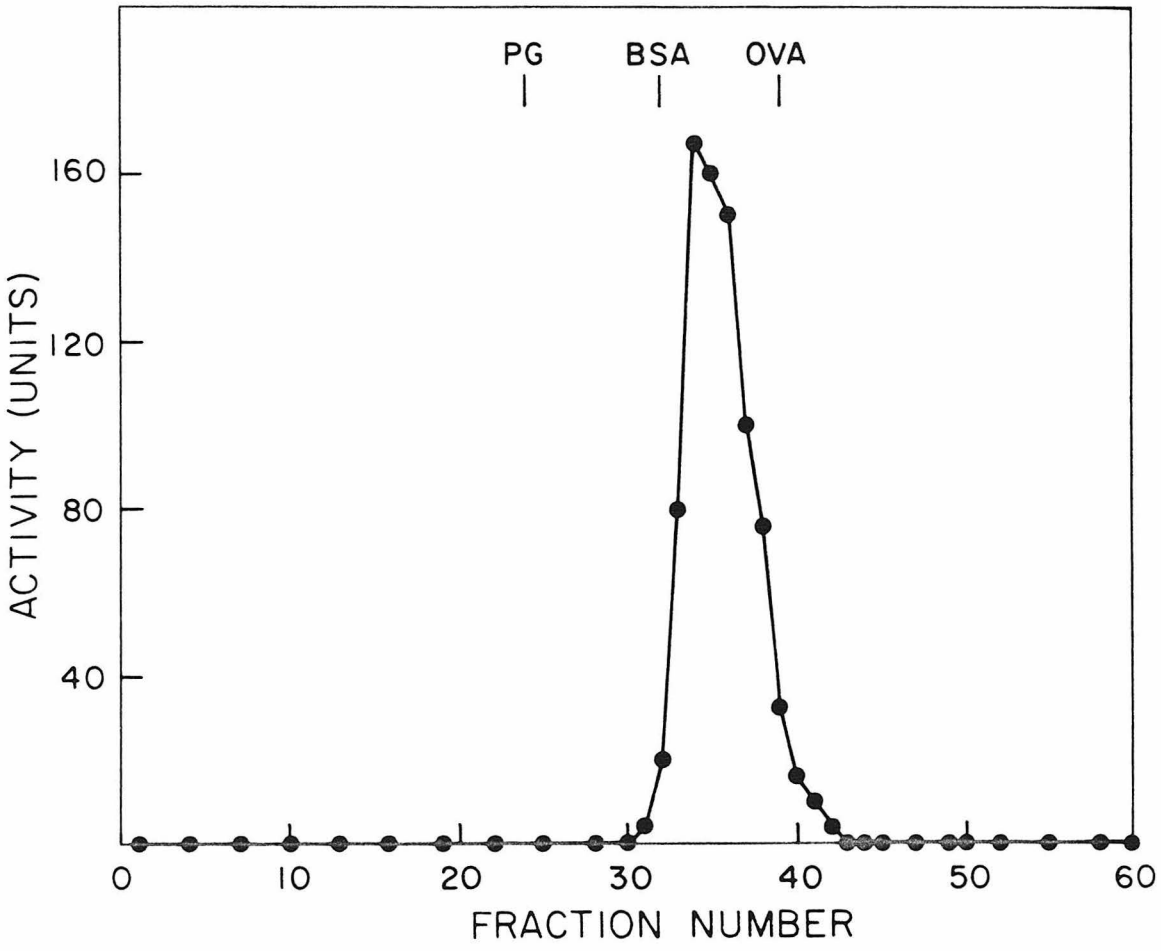
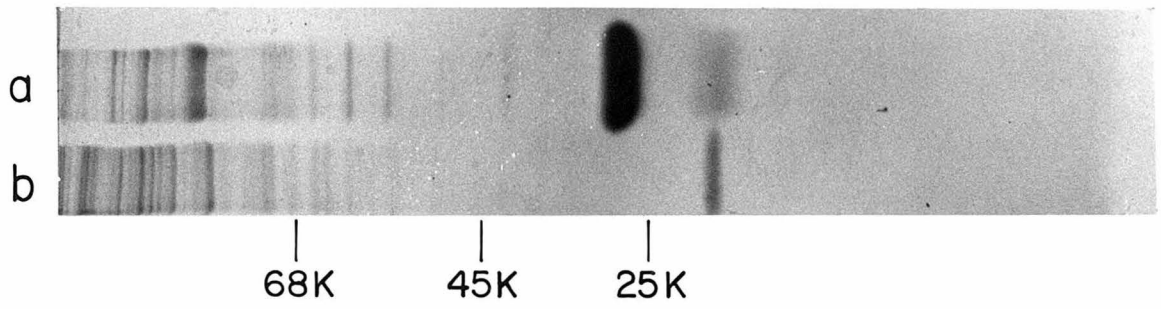

PG
BSA
OVA
160
120
80
40
ACTIVITY (UNITS)
0
10
20
30
40
50
60
FRACTION NUMBER

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 (pH 7.3) 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 urea, acid, and basic gels.


a
b
68K
45K
25K

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