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Purification and Properties of a Neutral Protease from Rat Liver Chromatin†

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ABSTRACT: Rat liver chromatin contains a neutral protease with a marked preference for chromosomal proteins as substrate. The enzyme has been purified about 700-fold. It has a molecular weight of 200,000 with two subunits. It is inhibited by phenylmethanesulfonyl fluoride and diisopropyl fluorophosphate. The enzyme requires divalent ions as activators. The isolated enzyme appears to be similar to that responsible for the endogenous degradation of chromosomal proteins. The susceptibility of the five histone fractions to proteolysis is dependent upon whether the histones are complexed with DNA. In the intact nucleohistone, four major histones are rather resistant to proteolytic attack while histone I is rapidly attacked. If histones are freed from DNA, all the histone molecules are attacked at about the same rate except histone I, which is degraded more slowly than the other histones.

It has been shown previously that isolated chromatin contains protease activity capable of degrading histones in chromatin (Furlan *et al.*, 1968; Panyim *et al.*, 1968; Garrels *et al.*, 1972). Much evidence suggests that association of histones with DNA prevents transcription (Shih and Bonner, 1970; Smart and Bonner, 1971). A possible role for chromatin-bound protease in removing histones from DNA during spermatogenesis has been suggested by Marushige and Dixon (1971). High substrate specificity is implied in this case since the protease must degrade histones without affecting protamines which replace histones in the sperm nucleus. Small heterogeneous acid soluble histone fragments were found in trout testis chromatin late in the transformation from nucleohistone to nucleoprotamine. It is also shown that histones become acetylated and phosphorylated during their replacement (Marushige and Dixon, 1969; Sung and Dixon, 1970; Candido and Dixon, 1972). Thus in spermatogenesis histones may be removed from DNA by proteolytic degradation; minor modifications may render the histones susceptible to such digestion. If the same hypothetical mechanism occurs in removal of histones during gene derepression, then the responsible protease would be an enzyme of biological significance. We have therefore purified this enzyme to homogeneity in an attempt to set the background for future studies on its role in gene regulation.

Materials and Methods

Frozen rat liver was from Pel-Freeze Biologicals, Inc.; poly(L-arginine) (MW 40,000) from Pilot Chemicals, Inc.; poly(L-lysine) (MW 5900) from Miles-Yeda Ltd.; salmon protamine sulfate, egg-white lysozyme, and bovine serum albumin from Sigma Co.; Bio-Rex 70 (200–400 mesh, sodium form), Bio-Gel A-50 (50–100 mesh), calcium phosphate gel, from Bio-Rad Lab; Sepharose 6B, Sephadex G-100, and QAE-Sephadex A-25 from Pharmacia Fine Chemicals; and human γ-globulin (fraction II) and ovalbumin (nonenzymic protein molecular weight markers) from Schwarz/Mann. *Escherichia coli* β-galactosidase was obtained from Worthington Biochemical Corp. Amicon PM-10 ultrafiltration membranes were from Amicon Corp. Dansyl chloride was from Pierce Chem. CO. Polyamide layer sheet was from Gallard-Schlesinger Chem. Corp.

Preparation of Nuclei. Nuclei were prepared by a modification of the method of Blobel and Potter (1966). The yield of nuclei based on the recovery of DNA was 50–70%.

In some instances as indicated in the text, the resulting

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nuclei were washed once with nonionic detergent by suspension in 0.5% (v/v) [Triton X-100–0.25 M sucrose–TKM[1] buffer (50 mM Tris (pH 7.5) at 20°–25 mM KCl–5 mM $MgCl_2$). After stirring at 4° for 30 min, the material was pelleted by centrifugation at 1500*g* for 10 min. The resulting pellet was washed twice with 0.25 M sucrose–TKM buffer by centrifugation as above. The chromatin was prepared from purified nuclei after washing with saline–EDTA as outlined in the Preparation of Chromatin section.

Isolation of Nuclear Membrane. These were isolated by the method of Kashnig and Kasper (1969).

Preparation of Chromatin. Chromatin was prepared from frozen rat liver as described by Elgin and Bonner (1970). Sucrose-purified chromatin was washed with 10 mM Tris (pH 8) and sheared in a Virtis homogenizer at 30 V for 90 sec. After centrifugation at 12,000*g* for 15 min, the supernatant was used as the starting material for the preparation of chromatin-bound protease.

Treatment of Chromatin with $PhCH_2SO_2F$ to Inactivate Endogenous Protease. Purified sheared chromatin was adjusted to 10 A_{260}/ml in 10 mM Tris (pH 8) and $PhCH_2SO_2F$ (50 mM of stock solution in isopropyl alcohol) was added to a final concentration of 1 mM. The resulting solution was stirred overnight at 4°, dialyzed against 0.1 mM $PhCH_2SO_2F$–10 mM Tris (pH 8) for 24 hr, and then dialyzed extensively against 10 mM Tris (pH 8). This procedure was effective in inactivating endogenous protease since subsequent incubation of chromatin for 8 hr at 37° followed by analysis of the disc electrophoretic patterns of histones and NHC proteins revealed no detectable breakdown.

Assay of Chromatin-Bound Protease Activity. Protease activity was assayed as previously described (Garrels *et al.*, 1972). The complete assay mixture contained the following components in a final volume of 0.2 ml: NaCl, 40 μmol; Tris (pH 8), 2.0 μmol; histones, 0.2 mg; and enzyme (0.02–0.4 μg of protein). After incubation in sealed test tubes for 20 hr at 37°, 0.5 ml of ninhydrin reagent was added to each assay sample. (Ninhydrin reagent is 0.4 g of ninhydrin, 80 ml of 95% ethanol, 1 g of $CdCl_2$, 10 ml of acetic acid, and 20 ml of water.) Similar assay mixtures but without addition of either substrate or enzyme serve as controls. The assay tubes were capped, placed in a boiling water bath for 4 min, and cooled quickly and absorbance at 506 nm was measured. One unit of protease activity is arbitrarily defined as the amount of enzyme that causes a change in absorbance at 506 nm of 0.01 above control values after 20-hr incubation.

There is no degradation of protein in assays in which the protease was previously treated with $PhCH_2SO_2F$ or Dip-F. Controls with added penicillin and streptomycin also show that microbial contamination does not affect the assay.

Purification of Chromatin-Bound Protease. All subsequent operations were carried out at 4°, unless otherwise mentioned. To purified sheared chromatin (20 A_{260}/ml in 10 mM Tris (pH 8)) was added crystalline sodium chloride with stirring to achieve a final concentration of 0.7 M. After additional stirring for 4 hr, the resulting solution was subjected to gel filtration by chromatography on Bio-Gel A-50 (4 × 100 cm column) equilibrated with and eluted by 0.7 M NaCl–10 mM Tris (pH 8). 10-ml fractions were collected. Fractions of the included protein peak were pooled and mixed with Bio-Rex 70 resin (2 mg of protein/g of resin) previously equilibrated with 0.4 M NaCl–10 mM Tris (pH 8), and the slurry was dialyzed against the same buffer. Columns were poured and washed with the same buffer. Nonadsorbed protein was collected and concentrated by ultrafiltration (Amicon PM 10 membrane). The concentrated protein solution was then applied to a Sepharose 6B column (2.5 × 116 cm) equilibrated with and eluted by 0.4 M NaCl–10 mM Tris (pH 8). Fractions containing the protease were pooled and mixed with calcium phosphate gel at the ratio of 1 mg of protein/ml of gel. After stirring for 15 min, the resulting slurry was centrifuged at 10,000*g* for 10 min. The pellet was collected and extracted with 0.4 M NaCl–10 mM sodium phosphate buffer (pH 8) and centrifuged as above. The resulting clear supernatant was dialyzed against 10 mM Tris (pH 7) and was applied to a QAE-Sephadex A-25 column (0.9 × 25 cm) previously equilibrated with 10 mM Tris (pH 7). The column was washed with the same buffer extensively and eluted with a linear salt gradient from 0 to 0.3 M NaCl–10 mM Tris (pH 7) in a total volume of 300 ml. The purified enzyme was eluted at 0.1 M NaCl concentration. (An aliquot was concentrated ten times for determination of protein concentration.)

Sucrose Gradient Centrifugation. Linear sucrose gradients (5–20%) in 0.1 M NaCl–10 mM Tris (pH 8) were prepared according to the method of Martin and Ames (1961). Human γ-globulin served as a standard (sedimentation coefficient 7.0 S, MW 160,000). Gradients were centrifuged at 116,000*g* in the SW 50 rotor for 10 hr at 3°. Samples were collected by punching a hole in the bottom of each tube with a needle and collecting drops; 8 drops per fraction were collected. Enzyme activity was assayed in each fraction and human γ-globulin was measured by A_{280}.

Preparation of Histones, Non-Histone Proteins, and Cytosol. Histones were prepared by acid extraction of purified sheared chromatin followed by precipitation of the extracted histones with ethanol (Bonner *et al.*, 1968). Non-histone proteins were prepared from rat liver chromatin according to the method of Van den Broek *et al.* (1973). Rat liver cytosol was prepared according to the method described by Kadenbach and Urban (1968). The cytosol was treated with 5 M urea–0.125 M NaOH overnight at 4° to inactivate endogenous proteolytic enzymes. The denatured cytosol was dialyzed exhaustively against 10 mM Tris (pH 7).

Purification of Histone I. Histone I was extracted from rat liver chromatin with 5% perchloric acid (Johns, 1964). To the extract 18% Cl_3CCOOH was added. The pellet was collected by centrifugation at 27,000*g*, washed twice with cold absolute ethanol (−20°), dissolved in 8% Gdn · HCl–0.1 M sodium phosphate buffer (pH 7), and applied to a Bio-Rex 70 column previously equilibrated with the same buffer. Protein was eluted with a linear gradient of 8–13% Gdn · HCl–0.1 M sodium phosphate buffer (pH 7). Purified histone I was eluted at the concentration of 8.8% of Gdn · HCl.

Isolation of nDNA from Rat Liver. Rat liver DNA was prepared from chromatin by the procedure of Wu *et al.* (1972).

Preparation of DNA–Histone I Complex. Reconstitution was performed by a modification of the procedure of Shih and Bonner (1970); 5 ml of DNA (1.85 mg/ml in 5 M

[1] Abbreviations used are: Dip-F, diisopropyl fluorophosphate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Gdn · HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; Na_2EDTA, disodium ethylenediaminetetraacetate; TKM, 50 mM Tris (pH 7.5)–25 mM KCl–5 mM $MgCl_2$.

TABLE I: Subnuclear Distribution of Protease Activity.

Expt No.	Nuclear Fraction	Total Protein (μg)	Total Units of Protease Activity	% of Total Protease Activity	Specific Activity (units/μg of Protein)
1	Whole nuclei	8150	3200 ± 160		0.40 ± 0.02
	Triton X-100 washed nuclei	7700	2920 ± 160		0.38 ± 0.02
	Nucleoplasm	1800	0	0	0
2	Nuclear membrane	850	391 ± 18	12	0.46 ± 0.02
	Chromatin	5500	2860 ± 100	88	0.52 ± 0.02
	Chromatin alone	95	50		
3	Nucleoplasm alone	35	0		
	Chromatin and nucleoplasm	130	50		

NaCl–5 M urea–10 mM Tris (pH 8)) was mixed by vigorous stirring with an equal volume of histone I solution (0.74 mg/ml in 10 mM Tris (pH 8)). The histone DNA mass ratio was thus 0.4. All the following steps were carried out at 4°. The mixture was dialyzed successively against a step gradient of NaCl of concentrations 2, 1.5, 1.0, 0.8, 0.6, 0.4, and 0.2 M; all solutions contained 5 M urea–10 mM Tris (pH 8). The resulting material was then dialyzed against 5 and 2.5 M urea–10 mM Tris (pH 8). Each of the above dialysis steps was for 4 hr. The urea was removed by exhaustive dialysis against 10 mM Tris (pH 8). The resulting complex was pelleted by centrifugation at 200,000*g* for 15 hr at 4°, yielding a transparent gel-like material with a histone I/DNA mass ratio 0.4 and a recovery of 70%.

N-Terminal Analysis. Qualitative N-terminal analysis was carried out by the method of Woods and Wang (1967).

Disc Gel Electrophoresis. Histones were analyzed on 15% polyacrylamide gels (pH 3.2) according to the method described by Panyim and Chalkley (1969). The nonhistone proteins were analyzed by sodium dodecyl sulfate disc gel electrophoresis using the Tris-glycine buffer system of King and Laemmli (1971). In the case of the purified enzyme it was necessary to use the entire yield (*ca.* 10 μg) of each single preparation for each single gel. We estimate that any contaminant constituting *ca.* 5% or more of the purified enzyme would have been detected.

Chemical Composition. Protein was determined by the method of Lowry *et al.* (1951), using crystal bovine serum albumin as standard. DNA was determined by measurement of absorbance at 260 nm (ϵ 20 l. cm^{-1} g^{-1}).

Results

Subnuclear Distribution of Protease Activity. The histone protease activity which has been reported by others (Garrels *et al.*, 1972) to be in either nuclei or chromatin of rat liver, is not a contaminant from cytoplasm. Washing nuclei with Triton X-100 to remove the outer nuclear membrane together with cytoplasmic contamination does not reduce the yield and specific activity of protease activity (Table I). Furthermore approximately 88% of this activity is localized in the chromatin fraction. The remaining activity is associated with the nuclear membrane fraction. The specific activity of protease is slightly higher in the chromatin than in the nuclear membrane fraction (Table I). Since no activity could be detected in the nucleoplasmic fraction, the possibility remained that this fraction contained an inhibitor of the protease activity. However, no evidence for the presence of an inhibitor was found by mixing experiments (Table I).

The protease activity is primarily bound to isolated chromatin. When chromosomal protein was released from DNA by treatment with increasing concentrations of sodium chloride, only 18% of protease activity was liberated at 0.3 M salt. Even at 2.5 M sodium chloride only 50% of protease activity was released. Routinely, the enzyme was released from chromatin by 0.7 M NaCl. This concentration yields the highest specific activity of soluble enzyme.

Purification of Chromatin-Bound Protease. Table II summarizes the steps used to purify chromatin-bound protease to homogeneity. The procedure resulted in a 705-fold purification over total chromosomal protein with a yield of

TABLE II: Purification of Chromatin-Bound Protease.

Purification Step	Volume (ml)	Protein Concn (μg/ml)	Total Protein (μg)	Activity (units/ml)	Total Activity (units)	Yield (%)	Specific Activity (units/μg of Protein)	Purification Factor
Total chromatin	600	950	570,000	500	300,000	100	0.52	1
Bio-Gel A-50	600	200	120,000	210	126,000	42	1.05	2.1
Bio-Rex 70	12	2500	30,000	3600	44,000	14.6	1.46	2.8
Sepharose 6B	38	64	2,430	540	20,500	6.8	8.44	16.2
Calcium phosphate gel	15	8	120	800	12,000	4	100	192.3
QAE-Sephadex	22	0.45	10	165	3,630	1.2	366.6	705.0

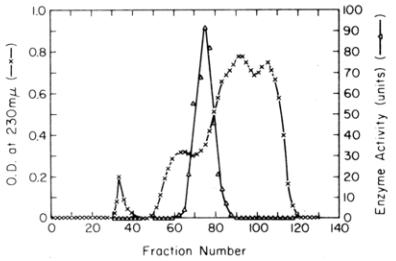

FIGURE 1: Chromatography of the protease activity on Sepharose 6B. Enzyme (44,000 units; 29,000 μg) in 12 ml of 0.4 M NaCl–10 mM Tris (pH 8) buffer was applied to the column (2.5 × 116 cm). Elution was with the same buffer; 3.8-ml fractions were collected; flow rate was adjusted to 20 ml/hr; all procedures were carried out at 4°.

activity of 1.2%. Standard methods were used. These included chromatography on Bio-Rex, Sepharose 6B (Figure 1), and QAE-Sephadex A-25 (Figure 2). The single most effective purification step, based on the criteria of both purification and recovery, was batchwise calcium phosphate gel adsorption (Table II).

Molecular Properties of Purified Protease. The molecular weight of the native purified enzyme was estimated to be approximately 200,000 by gel filtration and about 190,000 by sucrose gradient centrifugation (sedminentation coefficient, 7.8 S). A single symmetrical peak of protein was obtained when the purified protease was subjected to SDS disc electrophoresis in the presence of reducing agent (Figure 3). Densitometry indicated that over 95% of the applied protein migrated in this band, which had an estimated molecular weight of 100,000 relative to the mobilities of standards. It is thus concluded that the native enzyme is composed of two subunits with the same molecular weight. Upon the basis of the purification data (Table II), assuming a molecular weight of the active enzyme of 200,000 and that the rat haploid genome size is 1.8×10^{12} daltons (Britten and Davidson, 1971), isolated rat liver chromatin contains approximately 2.2×10^4 molecules of this enzyme per haploid genome. This estimate is subject to the caveat that upon enzyme purification no activation or inactivation has occurred.

Effect of pH, Ionic Strength, Temperature, Inhibitors, and Divalent Cations on Protease Activity. The purified enzyme, assayed with whole histone substrate, shows a pH optimum of 7.0 (Tris buffer), and has half-maximal activities at pH 9.0 and pH 6.0 (Figure 4). The enzyme is essen-

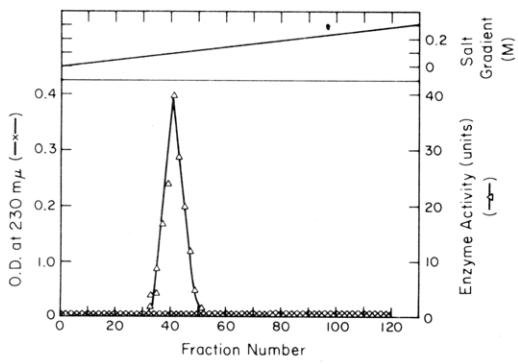

FIGURE 2: Chromatography of the protease activity on QAE-Sephadex A-25. Enzyme (12,000 units; 120 μg of protein) in 15 ml of 10 mM Tris (pH 7) was applied to the column (0.9 × 25 cm). Elution was with a linear sodium chloride gradient from 0 to 0.3 M in 10 mM Tris (pH 7); 2.5-ml fractions were collected; flow rate was adjusted to 15 ml/hr; all procedures were carried out at 4°.

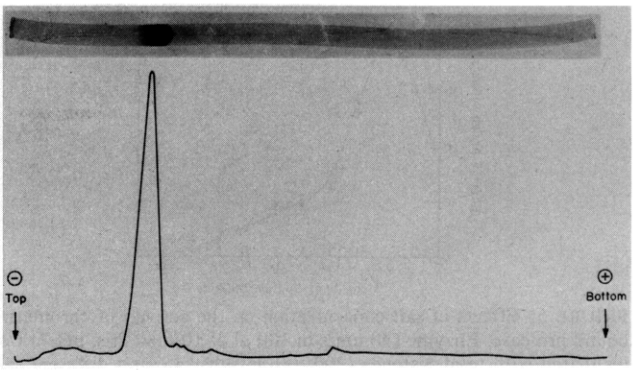

FIGURE 3: Analysis of purified protease by SDS acrylamide gel electrophoresis. The enzyme was analyzed in 10% acrylamide gels in the presence of 0.1% SDS. The major band represents over 95% of the total protein applied to the gel (0.3 × 10 cm).

tially inactive below pH 5.0 and above pH 10.0, presumably due to denaturation. The activity is maximal at 0.2 M sodium chloride, about twice the level observed at low ionic strengths (Figure 5). Above 1.0 M sodium chloride, the enzyme is inactive, but this effect is reversible. The enzyme is stable to storage for 4 months at −20°. However, 50% of the activity is lost after heating at 62° for 15 min and all activity is lost by heating at 70° for 15 min.

High concentrations of reducing agents (mercaptoethanol), and fairly low concentrations of heavy metals (Hg^{2+}, Cu^{2+}), are inhibitory, suggesting that both free sulfhydryl groups as well as disulfide linkages are important for activity and structure function relationships. The enzyme activity is partially inhibited by 2 M urea, but completely abolished by 1% SDS or 0.85 M guanidine hydrochloride. The serine hydroxyl group reagents, Dip-F and $PhCH_2SO_2F$, are potent inhibitors of the enzyme (0.1 mM + 1.0 mM, respectively); sodium bisulfite is also effective but requires higher concentrations (10 mM).

Since 1 mM EDTA was found to be inhibitory to protease activity, the effect of divalent metal ions on reactivation was investigated. Except for Mg^{2+}, all divalent metal ions tested were effective in reactivating the enzyme, Mn^{2+} being the most efficient (optimal concentrations being 6–8 mM). The inhibitory effect of Mg^{2+} is likely due to precipitation of the histone substrate under the assay conditions used.

Substrate Specificity. Table III summarizes the substrate specificity of the purified protease. Both histones and non-histone proteins are attacked preferentially compared to other substrates tested, including rat liver cytosol. It is clear that the enzyme prefers nuclear proteins as substrates. In addition it is interesting that poly(L-lysine) is a good

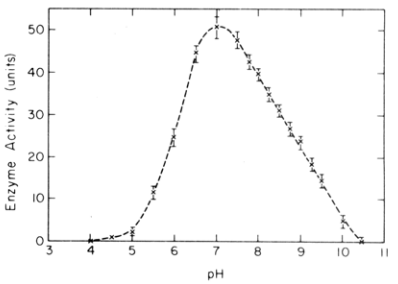

FIGURE 4: Effect of incubation pH on the protease activity; 50 units of enzyme in 0.2 M NaCl was incubated under different pH conditions. Enzyme activity assay was performed as given in the Materials and Methods section.

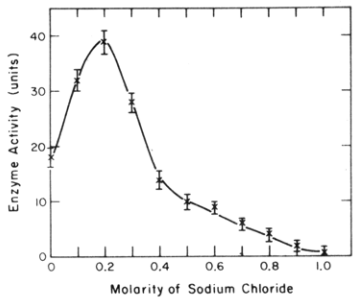

FIGURE 5: Effects of salt concentration on the activity of chromatin-bound protease. Enzyme (40 units in 100 μl of 10 mM Tris, pH 7) was incubated with total histones (200 μg) as substrate and different salt concentration. Total volume of incubation mixture was 200 μl. Activity assay was performed as given in the Materials and Methods section. Error bands are standard deviations calculated from eight experiments.

substrate for the enzyme, but poly(L-arginine) is a poor one. Denaturation of lysozyme and bovine serum albumin somewhat improved their abilities to serve as substrates.

The data from substrate saturation curves of enzyme activity on histones, non-histones, and cytosol of rat liver again indicate a strong preference of the enzyme for nuclear protein substrates. The K_m's for histones and non-histone proteins are 0.5 and 1 mg/ml, respectively.

The Difference in Digestion of Histones in Solution Compared to Histones Bound to DNA. The ability of the purified chromatin-bound protease to fragment histones, when used as substrates either in solution or complexed to DNA, was assayed by disc electrophoresis after incubation of samples with enzyme. As shown in Figure 6, when the substrates used were not complexed to DNA, all the histone molecules except histone I were attacked at about the same rate. If, on the other hand, the histones used as substrates were bound in native chromatin (endogenous protease being inactivated), histone I appeared to be most sensitive to degradation. This is in accord with the fact that in general histone I appears to be more accessible to the aqueous environment than are the other histones of native chromatin. Purified histone I was reconstituted to DNA by gradient dialysis. The breakdown products resulting from enzyme digestion of this complex were then compared to those resulting from enzyme fragmentation of histone I in solution and histone I in native chromatin (endogenous protease being inactivated). As shown in Figure 7, three principal products can be detected from fragmentation of histone I of native chromatin, while much more complex patterns of breakdown products were observed after digestion of histone I in solution or histone I reconstituted to DNA. It seems clear that histone I of native chromatin exhibits a conformation which favors limited and specific sites of attack by the enzyme and

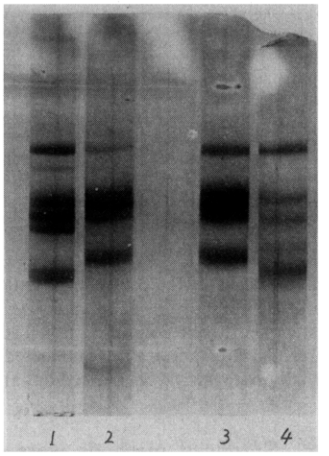

FIGURE 6: The differences in digestion of histones in solution compared with histones in chromatin. $PhCH_2SO_2F$ treated chromatin was digested with exogenous purified protease (gel 2) and without protease (gel 1) for 4 hr. Incubation at 37°, 0.04 M NaCl–10 mM Tris (pH 8), chromatin solution was 10 A_{260}/ml. $PhCH_2SO_2$-treated total rat liver histones were dissolved in 0.04 M NaCl–10 mM Tris (pH 8) and digested with exogenous purified protease (gel 4) and without protease (gel 3) for 4 hr at 37°. Standard histone urea gels. Migration is from the top (+) toward the bottom (−).

TABLE III: Substrate Specificity of Chromatin-Bound Protease.

Substrate	V_{max}/V_{max} of Histones
Histones	1.00
NHC protein	0.73
Cytosol[a]	0.02
Native chromatin-bound protein	0.54
L-Polylysine (MW 5900)	1.04
L-Polyarginine (MW 40,000)	0.08
Protamines	0.07
Casein	0.21
Lysozyme (in native form)	0
Lysozyme (denatured)[b]	0.104
BSA (in native form)[c]	0
BSA (denatured)[b,c]	0.07
Hemoglobin (denatured)[b]	0

[a] Rat liver cytosol was denatured in 0.125 M NaOH–5 M urea overnight, dialyzed extensively against 10 mM Tris (pH 7), and assayed as indicated in Materials and Methods. [b] The substrates were denatured in 0.125 M NaOH–5 M urea for 1 hr, neutralized, and assayed in 2.5 M urea–0.2 M NaCl–10 mM Tris (pH 7). [c] BSA, bovine serum albumin.

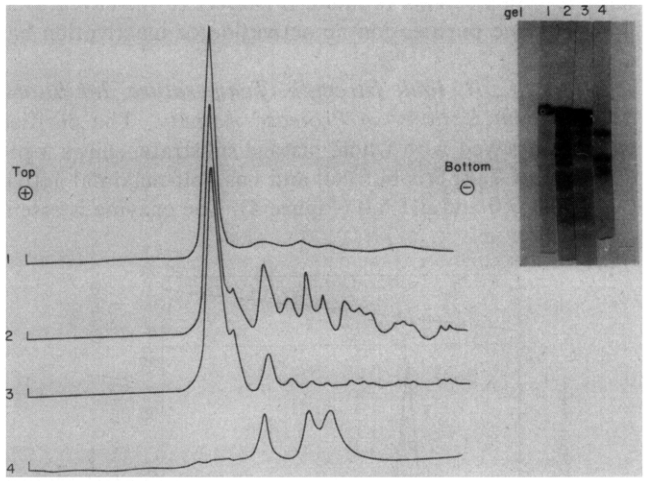

FIGURE 7: Degradation of histone I in solution, reconstituted to DNA, and in native chromatin by exogenous purified protease. Histone I in solution or histone I reconstituted to DNA (2 mg/ml) was digested with exogenous purified protease (50 units in 0.1 ml of 0.04 M NaCl–10 mM Tris (pH 8)) for 20 hr at 37°. $PhCH_2SO_2F$ treated chromatin 10 A_{260}/ml in 0.04 M NaCl–10 mM Tris, pH 8) was digested with exogenous purified protease (500 units/ml) for 20 hr at 37°. At the end of incubation, histone I and its degradation products were extracted with 5% perchloric acid and subjected to disc gel electrophoresis as indicated in Materials and Methods. (1) Intact histone I, (2) degraded histone I in solution, (3) degraded histone I reconstituted to DNA, (4) degraded histone I in native chromatin.

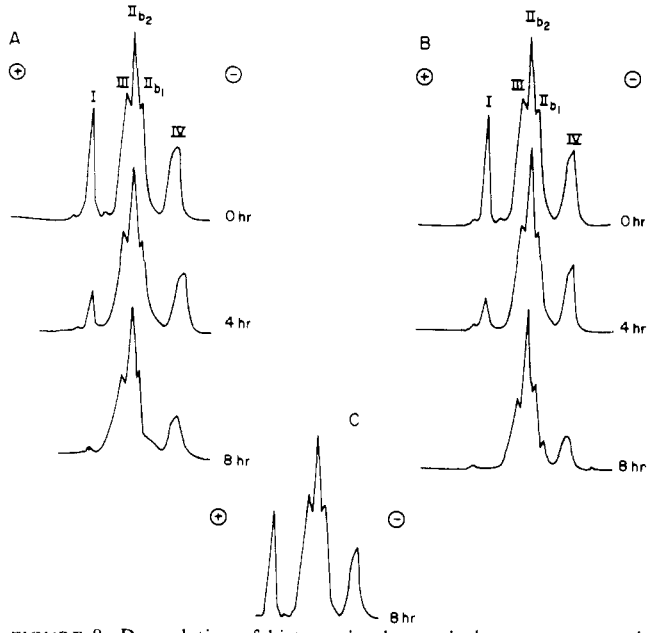

FIGURE 8: Degradation of histones in chromatin by exogenous purified protease (A) and endogenous protease (B). Incubation at 37°, 0.04 M NaCl–10 mM Tris (pH 8), chromatin concentration was 10 A_{260}/ml for 0.4 and 8 hr in each case. $PhCH_2SO_2F$ treated chromatin (in case A) was digested by exogenous purified protease (500 units/ml of chromatin). $PhCH_2SO_2F$ treated chromatin (C), without adding exogenous chromatin, was incubated for 8 hr to serve as control.

that the technique used to reconstitute histone I to DNA fails to achieve this conformation.

We have also tested whether the three principal products derived from histone I bound in native chromatin are the result of true proteolysis, rather than the effect of changes in charge distribution on histone molecules as a result, for example, of dephosphorylation or deacetylation. Both intact and appropriately degraded samples of histone I were dansylated by the method of Woods and Wang (1967). The interpretation of the results was facilitated by the observation (which we confirmed) that intact histone I possesses no free N-terminal groups (Bustin *et al.*, 1969). On the other hand, degraded products of histone I from native chromatin showed two new N-terminal groups (lysine and alanine). This result argues forcibly that the faster moving electrophoretic bands are the result of true proteolysis.

Similarity of Purified Protease to Endogenous Chromatin-Bound Protease Activity. Since only 1.2% of the total chromatin-bound protease activity was recovered after purification of the enzyme to homogeneity (Table II), the possibility remained that the purified enzyme might not be representative of the major activity bound to chromatin. To test this possibility, native rat liver chromatin samples were incubated at 37° for various periods of time, after which histones and non-histone proteins were isolated and separated by disc electrophoresis. The same assay procedure was used for purified protease acting on chromatin. In this case the endogenous activity of the chromatin was first inactivated by $PhCH_2SO_2F$. As shown in Figures 8 and 9, the orders of breakdown of various chromosomal proteins catalyzed by the purified enzyme were essentially the same as those catalyzed by the endogenous activity. We conclude that the purified protease is representative of the endogenous activity of chromatin.

Discussion

The protease we have isolated is somewhat different from

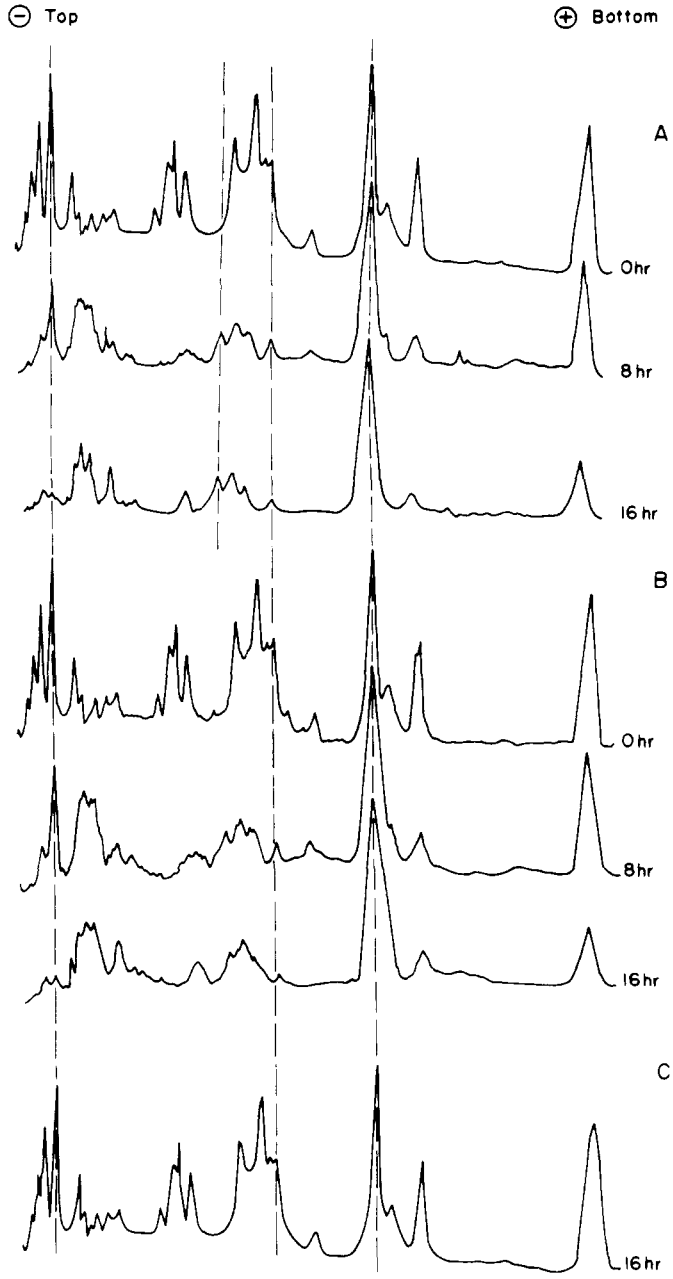

FIGURE 9: Degradation of NHC proteins in chromatin by endogenous protease (A) and exogenous protease (B). Incubation at 37°, 0.04 M NaCl–10 mM Tris (pH 8), chromatin was 10 A_{260}/ml for 8 and 16 hr in each case. $PhCH_2SO_2F$ treated chromatin (in case B) was digested by exogenous purified protease (500 units/ml of chromatin).

the neutral protease isolated from calf thymus by Furlan and Jericijo (1968). For example, the molecular weight of our enzyme is about nine times larger than theirs; its optimal pH is 7 instead of 7.8; our enzyme needs divalent ions as activator and is inhibited by mercuric ion. However, there are some properties shared by both enzymes, for example, enzyme activity is salt dependent, thermolabile, and inhibited by Dip-F; histone I is the first protein attacked by the enzyme when histone I is bound to chromatin, but once it is dissociated from chromatin, it is the last to be degraded.

It is clear that the manner in which histones are attacked by the enzyme is dependent on whether or not they are complexed to DNA. This point was emphasized earlier by Bartley and Chalkley (1970), who also studied endogenous chromatin-bound protease activity. That histone I in chro-

matin is more exposed to the aqueous environment than the other histones is suggested not only by its rapid attack by chromosomal protease but also by its rapid formaldehyde fixation to DNA (Brutlag *et al.*, 1969), its extraction by low salt concentration, (Ohlenbusch *et al.*, 1967) and by ORD studies on α helicity (Tuan and Bonner, 1969). Histones II, III, and IV are protected from attack by the enzyme when complexed to chromatin, yet rapidly degraded when free in solution, indicating that these proteins are involved in much more intimate association with DNA than is histone I.

Because the enzyme purified in this study yields identical breakdown products to those produced by the endogenous chromatin-bound activity, we believe the two are one and the same enzyme. The fact that the enzyme prefers nuclear protein substrates suggests a physiological role in the turnover of nuclear proteins. One such role could be in the turnover of non-histone proteins, which have been shown to have high turnover rates (Dice and Schimke, 1973). The pronounced susceptibility of histones to attack by the enzyme, is not, however, compatible with the low turnover rate of histones in cell nuclei. At least three possibilities could account for this observation: (1) the enzyme may be activated by chromatin purification, (2) the enzyme may be inhibited by a nucleoplasmic inhibitor, although no evidence for this possibility was found, and (3) the enzyme might be localized in specific sites in nuclei and, after nuclear lysis, become redistributed.

Other work in our laboratories (Garrard and Bonner, 1974) directly implicate the enzyme, which we have purified, in the removal of histones from DNA in the course of gene derepression. The nature of the signals which cause only histones associated with particular nucleotide sequences is an obvious sequel to our work.

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