Histone-Dependent Reconstitution and Nucleosomal Localization of a Nonhistone Chromosomal Protein: The H2A-Specific Protease†

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ABSTRACT: We have described earlier a chromatin-bound protease with unique specificity for histone H2A [Eickbush, T. H., Watson, D. K., & Moudrianakis, E. N. (1976) *Cell (Cambridge, Mass.)* 9, 785-792]. In the present study, we explore the nature of interactions that form and stabilize the enzyme–chromatin system by using the activity of the protease to monitor its binding to DNA and DNA–histone complexes. During salt extraction of chromatin, the protease is released at an ionic strength between that required for the extraction of the slightly lysine-rich histones (H2A and H2B) and the arginine-rich histones (H3 and H4). The reassociation of this nonhistone protein to DNA has an absolute requirement for the H3–H4 tetramer and is only enhanced by the H2A–H2B dimer in the presence of the tetramer. We believe that the binding of the enzyme onto DNA requires some histone-elicited compaction of the helix. We have also examined the distribution of this enzyme within the chromatin fiber by isolating pools of monomer nucleosomes from micrococcal nuclease digests of 0.6 M NaCl extracted chromatin and from reconstituted DNA–protein complexes. The H2A–protease is found with these monomer nucleosome pools, and no activity can be detected in the low molecular weight products released during the digestion. Thus, by virtue of its extraction characteristics from chromatin and its association with isolated nucleosomes, this nonhistone protein exhibits properties hitherto assigned only to the inner histones.

Recent investigations on chromatin structure have emphasized the role of the histone proteins, while relatively little attention has been paid to the role of the nonhistone proteins [for review, see Elgin & Weintraub (1975)]. Two general factors have contributed to the neglect of this major component of chromatin. First, workers using a variety of probes to study chromatin structure have concluded that the histones alone are responsible for the generation of nucleosomes and for eliciting the primary compaction of the DNA (Germond et al., 1975; Camerini-Otero et al., 1976; Camerini-Otero & Felsenfeld, 1977) by allowing the helix to express its intrinsic property of forming a left-handed supercoiled fiber (Eickbush & Moudrianakis, 1978a). Second, the heterogeneity of the nonhistone proteins has hindered rapid progress in this area. Differences in their electrophoretic mobilities in polyacrylamide gels provided the only characterization of most nonhistone proteins, and to date, no objective agreement has been reached as to how true nonhistone proteins could be differentiated from possible cytoplasmic contaminants (Johns & Forrester, 1969; Jackson, 1976). Obtaining purified nonhistones became a prerequisite for further study, and some analyses utilizing purified proteins, such as those examining the HMG–nonhistones (Yu & Spring, 1977; Yu et al., 1977; Weisbrod et al., 1980), have been initiated.

Following the original observations of Hewish & Burgoyne (1973) as extended subsequently by Noll (1974), it is generally accepted today that the nucleosome (200 bp[1] of DNA plus two each of the histones H2A, H2B, H3, and H4) is the basic repeating structural unit of the chromatin fiber (Kornberg, 1974). Several reports have demonstrated the presence of nonhistone proteins in preparations of monomer nucleosomes, but none has successfully demonstrated that a defined nonhistone can be found exclusively with *isolated core nucleosomes*.

We have recently purified a nonhistone protein with a defined activity: the restricted cleavage of histone H2A. We have determined the exact site of cleavage of H2A and have demonstrated that this nonhistone is not derived from trivial cytoplasmic contamination (Eickbush et al., 1976). The absence of cleaved H2A from freshly isolated calf thymus chromatin, or with chromatin incubated at low ionic strength, however, suggests that, in chromatin, the protease may be somehow sequestered. For the present report, we have examined the nature of the association of the protease with the other components of chromatin as revealed by a variety of extraction procedures and have found that the binding of the protease to DNA is similar to that of the core histones. We have also reconstituted complexes between the protease, variable amounts of defined histones, and pure DNA and monitored the binding of the enzyme to DNA by following the restricted cleavage of H2A. We will show that there is a requirement for histones to be present in the reconstitution system for proper binding of the enzyme to DNA and that the arginine-rich histones have the greatest effect on the binding of the protease to the reconstituted nucleohistone. Finally, we determined the relative distribution of the protease along the native and reconstituted chromatin fiber. Extensive micrococcal nuclease digestion of both types of complexes led to the recovery of the protease exclusively with the monomer nucleosome pool while no activity was detected in the pool of internucleosomal fragments.

Materials and Methods

Preparation of Chromatin, Histone, and Histone Complexes. Calf thymus chromatin and monomer histones were prepared as described previously (Eickbush et al., 1976). The histone octamer was prepared as described recently (Eickbush & Moudrianakis, 1978a). The H3–H4 tetramer and the H2A–H2B dimer were obtained by pH 5 dissociation of pure octamer and subsequent fractionation of the octamer subunits

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; bp, base pairs; cH2A, cleaved H2A.

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on a Sephadex G-100 column (2.3 × 215 cm), equilibrated with 2 M NaCl and 10 mM NaH_2PO_4 (pH 5.0).

Preparation of the H2A–Protease. A detailed presentation of the purification of the protease will be presented in a forthcoming communication, which also characterizes several properties of the enzyme. For these studies, we have used partially purified enzyme, obtained by fractionation of a NaCl–urea extract of calf thymus chromatin on a Sephadex G-75 column, equilibrated with 2 M NaCl and 10 mM glycine (pH 9.0). This single chromatographic step allowed us to remove most of the histones present in the chromatin extract from the enzyme. No other proteolytic activity is detected in this fraction under the assay conditions used for this study.

Chromatin Extractions. NaCl. Calf thymus chromatin (at 1 mg/mL) was extracted with an equal volume of NaCl in 20 mM NaH_2PO_4 (pH 5.5) to yield the desired NaCl concentration. Aliquots of the supernatants from a Ti50 ultracentrifugation were analyzed for histone composition by Na-$DodSO_4$–polyacrylamide electrophoresis. Determination of the relative concentration of each histone was made by scanning the gels at 560 nm and calculating the area under each peak using a Dietzgen planimeter. For the determination of histone concentration, extractions also contained 1 mM PMSF (phenylmethanesulfonyl fluoride) to prevent proteolysis. The release of the H2A–protease was monitored by cleavage of ^{3}H-labeled H2A (see below).

NaCl–Urea. Chromatin (at 2 mg/mL) was mixed with increasing concentrations of NaCl and a constant amount of urea to yield chromatin at 0.5 mg/mL at the desired concentration of NaCl and 2.0 M urea. Extracts were processed as described for the NaCl extractions.

Preparation of DNA and of Reconstituted DNA–Protein Complexes. DNA was purified from the pellets of the salt-extracted [2 M urea, 0.05 M $NaHSO_3$ (pH 5.0), and 2 M NaCl] chromatin. Pellets were resuspended in 1 × SSC at less than 2 mg/mL DNA and digested with RNase (50 μg/mL) and Pronase (100 μg/mL) before being extracted with redistilled phenol (previously equilibrated with SSC). After three or more phenol extractions, the DNA was reextracted (at least 3 additional times) with $CHCl_3$–isoamyl alcohol (24:1). This material was then precipitated with ethanol and resuspended at 1 mg/mL in 1 × SSC and 1 mM EDTA and stored over $CHCl_3$ until needed. DNA preparations had an A_{260}/A_{230} ratio greater than 2.4. For our experiments, this DNA was sheared in a Lourdes homogenizer (Lourdes Instrument Corp., Brooklyn, NY) for 10 s at 100% output. This method of preparation allows us to obtain a pool of DNA of average molecular weight of 30×10^6, as estimated from the relative mobilities of the sheared DNA and purified λ DNA on 1% agarose gels (Hayword, 1972).

For formation of most of the reconstituted complexes, DNA and all proteins were dialyzed separately against 2 M NaCl, 10 mM NaH_2PO_4 (pH 5.0), before mixing the protease, and 0.2 mg of each histone to be tested with 1.0 mg of DNA. The mixtures of proteins and DNA remained on ice for at least 1 h before being dialyzed overnight against 0.6 M NaCl or 0.3 M NaCl in 10 mM NaH_2PO_4 (pH 5.0). The resultant DNA–protein complexes were separated from free protein by exclusion chromatography on Bio-Gel A-50 columns (1.25 × 22 cm) at 4 °C equilibrated with the same buffer as that used for dialysis. When either histone H3 or H4 was used in the absence of the other, the initial dialysis buffer was 2 M urea, 2 M NaCl, and 10 mM NaH_2PO_4 (pH 5.0). These mixtures were then dialyzed for 1 h against 2 M NaCl and 10 mM NaH_2PO_4 (pH 5.0) and then processed as described above.

Chromatin Digestion. Nuclei obtained by our normal procedure were resuspended in 0.01 M Tris-HCl and 1 mM EDTA (pH 8.0) and made 0.6 M NaCl by the addition of solid NaCl. After swelling at 4 °C for 8–10 hours, the resulting chromatin gel was centrifuged at 15000 rpm in an SS34 rotor and transferred to 0.65 M NaCl, 10 mM Tris–cacodylate (pH 7.2), and 0.7 mM EDTA as described by Tatchell & Van Holde (1977). This suspension was allowed to stir slowly overnight, followed by centrifugation and resuspension in the same medium. After at least 1 h, this solution was centrifuged and resuspended in 10 mM Tris–cacodylate and 0.7 mM EDTA. This low ionic strength wash was repeated 1 time. The chromatin so prepared was additionally washed 3 times with 10 mM Tris (pH 8.0) and adjusted to 0.4–0.5 mg/mL DNA. The chromatin was made 0.16 mM $CaCl_2$ and equilibrated at 37 °C before the addition of micrococcal nuclease (Sigma; approximately 15 A_{260} units/mL as defined by Sigma). The digestion was stopped after 1 h (at least 35% acid solubility) by making the solution 1.6 mM EDTA (pH 7.5) and rapidly cooling to 0 °C.

Isolation of Monomer Nucleosome Pool. Aliquots of the total chromatin digest were fractionated on a linear 5–20% sucrose gradient (in 1 mM cacodylate, 1 mM EDTA (adjusted to pH 6.5 with Tris), and 0.4 M NaCl) by centrifugation in a SW25.1 rotor at 22000 rpm for 38 h at 4 °C. Fractions were collected from the bottom of the tube and monitored by A_{260}.

Pools were concentrated by vacuum dialysis and repurified on gradients (in the above buffer without NaCl) utilizing a SW50.1 rotor at 42000 rpm for 6 h. This material was used for all experiments with native calf thymus monomer nucleosomes.

Digestion of DNA–Octamer Complexes. Before digestion, DNA–octamer complexes were dialyzed extensively against 1 mM Tris-HCl (pH 8.0). Digestion was carried out as described for chromatin, except that the DNA concentration and total digestion time were cut in half.

Preparation of Soluble Chromatin. Calf thymus nuclei were prepared for digestion as previously described (Shaw et al., 1974). So that soluble chromatin could be obtained, nuclei were incubated for 3.5–7 min at 37 °C in the presence of micrococcal nuclease (Sigma; at 25–40 A_{260} units/mL). After termination of the reaction by the addition of EDTA to 1.7 mM, particles larger than trimer nucleosomes were isolated on sucrose gradients (prepared as described above for the isolation of monomer nucleosomes, but sedimentation was only for 14 h). Prior to use in mixing experiments with brain monomer nucleosomes, both pools were dialyzed against 10 mM Tris-HCl (pH 8.0) and concentrated by vacuum dialysis. Equivalent amounts (A_{260} units) of these pools were mixed and allowed to remain at 0 or 37 °C for 60 min in the presence or absence of 0.16 mM $CaCl_2$. Mixtures were then resolved into monomer nucleosomes and oligonucleosomes by sedimentation on sucrose gradients. Pools were analyzed for proteolytic activity by the cleavage of ^{3}H-labeled H2A (described below).

Electrophoresis of Proteins and DNA. Histones were analyzed on Na$DodSO_4$–polyacrylamide gels, prepared and run as described (Eickbush et al., 1976). DNA samples were purified for electrophoresis as described (Noll, 1974) and run on a 6% polyacrylamide gel, using the buffer system of Loening (1967) with a Bio-Rad vertical slab gel apparatus. The gel was run for 4 h at 10 V/cm and, after staining for 30 min with 0.5 μg/mL ethidium bromide, was photographed through a red filter using Polaroid Type 665 positive–negative film.

Protease Assays. Gel Analysis. Chromatin, 0.6 M NaCl extracted chromatin, DNA–protein complexes or isolated pools from nuclease digestions were dialyzed against 10 mM Tris and 1 mM EDTA (pH 8.0). Aliquots for analysis were mixed with an equal volume of 4.0 M NaCl and 20 mM Tris-HCl (pH 9.0), resulting in a final DNA concentration of 0.125–0.25 mg/mL and ionic strength of 2.0 M NaCl and pH 8.65. After the desired times of incubation at 37 °C, aliquots were made 0.4 N H_2SO_4, followed by pelleting of the precipitated DNA and liberation of the extracted histones by centrifugation at 8000 rpm in a SS34 rotor. Histones were analyzed by Na-DodSO$_4$–polyacrylamide electrophoresis, and the percentage of the H2A molecules cleaved was estimated as described (Eickbush et al., 1976).

^{3}H-Labeled H2A Cleavage Analysis. H2A was labeled by the procedure of Means & Feeney (1968) using [^{3}H]NaBH$_4$ (New England Nuclear). Aliquots of samples to be tested for proteolytic activity were made 2.0 M NaCl and incubated with ^{3}H-labeled H2A (200 μg/mL) at 37 °C for the desired length of time. The mixture was made 1 mg/mL BSA and 10% Cl_3CCOOH and centrifuged at 8000 rpm in a SS34 rotor to stop the reaction. Counts released into the supernatant were directly related to the amount of cH2A produced during the incubation (established by comparing time courses of tritiated pentadecapeptide release and cH2A production). In these assays, there is a linear relation between the amount of enzyme added and cH2A produced up to the point of 50% substrate conversion. All of the experiments reported here were carried out under conditions yielding less than 30% conversion of H2A to cH2A.

Quantitative Determinations. Micrococcal nuclease concentration was determined by the procedure of Lowry et al. (1951), but modified as described previously (Rubin & Moudrianakis, 1972). Histone concentration was determined by the biuret procedure (Zamenhof, 1957), using BSA (Sigma, fraction V) as a standard, and the values from this assay were comparable with those from total nitrogen determination.

The DNA concentration was determined spectroscopically, by measuring the A_{260}, using extinction coefficients of 22 cm^2 mg^{-1} at 260 nm for pure DNA and 20 cm^2 mg^{-1} at 260 nm for DNA–protein complexes and chromatin.

Results

The Affinity of the Protease for DNA Differs from That of Most Nonhistone Proteins. Most nonhistone proteins are either removed from chromatin by 0.6 M NaCl or remain bound to the DNA even after all the histones have been removed by 2.0 M NaCl. No significant amount of the protease is extracted from chromatin by 0.6 M NaCl (Figure 1A). It is only after a large percentage of H2A and H2B have been released that the enzyme is extracted. Since a small fraction of the H2A remains bound to the chromatin until the protease is released, one could suggest that the protease is solubilized as an enzyme–substrate complex. During the release of the arginine-rich histones H3 and H4, no additional enzyme is extracted.

Since ionic interactions between components of chromatin should be greatly reduced or abolished by 0.6 M NaCl and because the protease remains bound to chromatin in 0.8 M NaCl, some nonionic interaction(s) between the enzyme and chromatin must be present. We have extracted chromatin with increasing concentrations of NaCl in the presence of constant 2.0 M urea, conditions known to facilitate extraction of the core histones by disrupting nonionic interactions (Bartley & Chalkley, 1972). It can be seen (Figure 1B) that while the order of release remains unaffected by this reagent (compare,

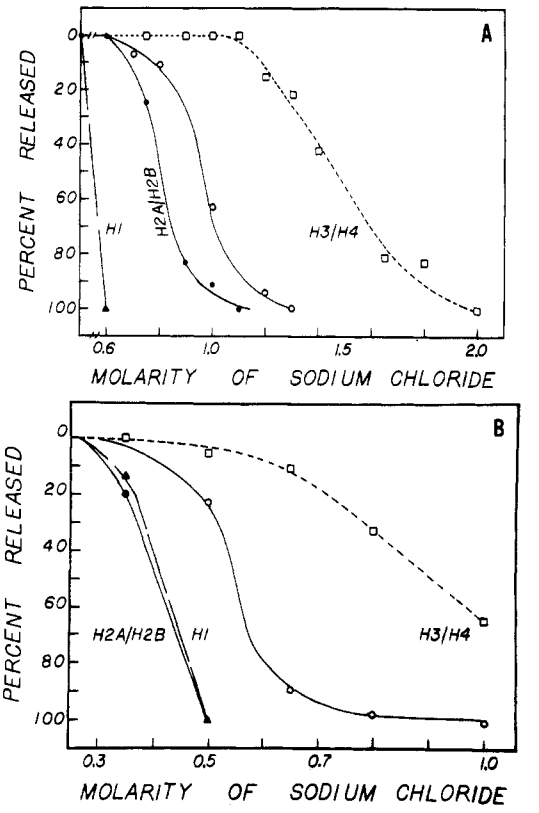

FIGURE 1: Relative extraction of the H2A–protease and the histones from calf thymus chromatin. (A) NaCl extraction: Chromatin was extracted with increasing sodium chloride. Aliquots of the extracts were analyzed for histone composition and for proteolytic activity after sedimentation to remove the DNA. (▲) H1; (●) H2A + H2B; (□) H3 + H4; (○) proteolytic activity. (B) NaCl/urea extraction: Chromatin was extracted with a constant amount of urea (2.0 M) and increasing concentrations of NaCl, as indicated. Symbols as described in (A).

Figure 1A), the extraction of the protease is preferentially facilitated. The complete solubilization of the H2A before significant amounts of the protease are released argues against their extraction from chromatin as an enzyme–substrate complex under these conditions.

Thus, the protease, like the core histones, is bound to chromatin through ionic and nonionic interactions and exhibits extraction characteristics like those of the inner histones rather than those of H1 and most nonhistone proteins.

The Protease Can Be Reconstituted onto DNA. No significant amount of the H2A–protease is extracted from calf thymus chromatin by 0.6 M NaCl (Figure 1A). We thus decided to use this property as a *first* operational criterion for proper binding of the protease to DNA; that is, upon "proper" reconstitution, no enzyme should be removed by exposure of the DNA–protein system to 0.6 M NaCl.

A model reconstituted chromatin complex was formed by exposing native chromatin to 2.0 M NaCl followed by dialysis to 0.6 M NaCl in order to allow rebinding of most of the extracted proteins. The extraction characteristics of the protease from this chromatin reconstituted complex were compared to those of native chromatin. Bio-Gel A-50 exclusion chromatography was used to separate DNA–protein complexes from free protein. The results of these extractions are presented in Table I, where it can be seen that the amount of activity found associated with the 0.6 M NaCl extracted "reconstituted" chromatin is essentially identical with that present in 0.6 M NaCl extracted native chromatin. It can be concluded then that upon reassociation of the chromatin components, all the protease present in the original native

Table I: Protease Extraction from Total Chromatin and from a Reconstituted System Containing All Chromatin Components[a]

system	extracted with NaCl (M)	protease act. (cpm)	cpm/A_{260}	act. (%)
total chromatin	0.6	1667	640	100
(control)	0.8	1744	614	96
	1.0	0	0	0
reconstituted	0.6	1637	695	109
chromatin	0.8	1649	627	98
	1.0	0	0	0

[a] The salt extraction characteristics of the protease from control (native) and reconstituted chromatin (chromatin that had been treated with 2.0 M NaCl and dialyzed down to 0.6 M NaCl) are compared. After the control chromatin was mixed with an equal volume of 1.2 M NaCl, aliquots of control and reconstituted chromatin were mixed with an equal volume of salt to achieve the desired sample extraction conditions. Protease activity associated with the DNA-protein complex (2.14–2.84 A_{260}/cm) excluded from a Bio-Gel A-50 column was measured by cleavage of ^{3}H-labeled H2A (soluble radioactivity measuring pentadecapeptide release), after the buffer conditions were adjusted to 2.0 M NaCl, pH 8.3–8.4, by the addition of an equal volume of NaCl in 20 mM Tris (unbuffered). Incubation was at 37 °C for 2.5 h.

chromatin can be bound reversibly and in the proper manner.

Reconstitution of the Protease Requires Only the Core Histones. Could the protease be bound to the DNA in the absence of all other nonhistone proteins? Using partially purified protease and pure calf thymus DNA which had been sheared to an average of 30×10^6 daltons, we have examined the effect of the isolated histone octamer (Eickbush & Moudrianakis, 1978b) upon the reconstitution of the protease. For all of our experiments, we have added an amount of protease activity which is less than or equal to that found with isolated calf thymus chromatin. As can be seen in Table II (experiment 1b), when the protease, the histone octamer, and DNA are the only components present in the reconstitution mixture, the enzyme binds properly to the DNA. The DNA–protein complex that is excluded from the Bio-Gel A-50 column has essentially the same specific activity as the unfractionated total reconstituted mixture. As with native chromatin, the protease was bound in reversible manner with the DNA–histone octamer system. The most significant finding of this experiment is that very little enzyme remains bound to the DNA at 0.6 M NaCl when no histone was present in the mixture (experiment 1a).

While attempting to purify the enzyme, we observed that the addition of a variety of proteins, such as histone H1, BSA, and α-casein, extended the lifetime of the enzyme. Therefore, to afford better recovery of the input activity, we decided to incorporate histone H1 into all reconstitution mixtures. Most of our reconstitution experiments never expose the mixtures to an ionic strength less than 0.6 M; thus, the H1 will never be associated with the DNA. The data presented in Table II (experiments 2a and 2b) demonstrate that although the addition of the histone H1 to the samples results in better recovery of the input activity, successful reconstitution of the protease to DNA again requires the presence of the histone octamer.

The H3–H4 Tetramer Is the Key Element Required for Successful Protease Reconstitution. It has already been shown that the histone octamer is composed of one H3–H4 tetramer and two H2A–H2B dimer subunits (Eickbush & Moudrianakis, 1978b; Godfrey et al., 1980). It is important, then, to determine whether the reassociation of the enzyme with DNA requires the entire core histone complement or a specific subunit of the histone octamer. When the H3–H4 tetramer,

Table II: Reconstitution of the H2A-Protease to DNA[a]

proteins tested	extracted with NaCl (M)	protease act. (cpm)	cpm/A_{260}	act. (%)
experiment 1				
(a) no additions		415	247	27
	0.6	0	0	0
	0.8	0	0	0
	1.0	0	0	0
(b) histone octamer		1581	934	100*
	0.6	2059	1246	133
	0.8	1520	916	98
	1.0	0	0	0
experiment 2				
(a) H1		1486	813	81
	0.6	57	34	4
(b) H1 + histone		1852	1000	100*
octamer	0.6	1482	792	79
(c) H1 + H3/H4		1859	1015	102
tetramer	0.6	1392	739	74
experiment 3				
(a) H1		2182	1250	90
	0.6	104	59	4
(b) H1 + H2A/H2B		2328	1384	100*
dimer$_{(n)}$	0.6	2458	1342	97
(c) H1 + H2A/H2B		2169	1221	88
dimer$_{(r)}$	0.6	128	72	5

[a] Reconstituted DNA–protease complexes in the presence or absence of other histones were prepared as described under Materials and Methods. For examination of the salt extraction characteristics of the protease (experiment 1), aliquots of the original sample (in 0.6 M NaCl) were mixed with an equal volume of the appropriate concentration of NaCl (in 10 mM NaH_2PO_4, pH 5.0); after standing on ice for at least 1 h, extracted proteins were separated from DNA–protein complexes by Bio-Gel A-50 exclusion chromatography on columns equilibrated with the same salt concentration as that of the extract. For determination of the amount of enzyme bound to DNA after dialysis to 0.6 M NaCl (experiments 2 and 3), the activity associated with the Bio-Gel A-50 excluded DNA–protein complex (bound enzyme) was compared to that of the unfractionated sample in each of the three experiments (set to 100%, marked by asterisk). Unfractionated and excluded complexes were adjusted to similar DNA concentrations (1.60–1.88 A_{260}/cm) and assayed for protease as described in Table I, except that the incubations were conducted at 37 °C for 6.5 (experiment 1), 10 (experiment 2), or 4 h (experiment 3). H2A/H2B dimer$_{(n)}$ and H2A/H2B dimer$_{(r)}$ stand for native dimer and reconstituted dimer, respectively.

histone H1, and the protease are mixed with DNA, the resulting DNA–protein complex contained essentially the same amount of enzyme as had been found with the DNA–octamer complex (Table II; compare experiments 2c and 2b). No other proteins besides histone H3 and histone H4 can be detected upon electrophoresis of the tetramer fraction used for these studies. Thus, the H3–H4 tetramer alone is sufficient to afford efficient reconstitution of the protease.

Some protease can also be bound to the DNA when the H2A–H2B dimer preparation alone is used for reconstitution. However, the enhancement of the protease binding is very dependent upon the method of preparation of the dimer. The H2A–H2B dimer obtained from the pH 5 dissociation procedure ("native dimer") facilitates the binding of the protease to DNA (Table II, experiment 3b). Because this type of preparation may contain low levels of contaminating tetramer, we prepared pure H2A–H2B dimer by direct mixing of homogeneous H2A and H2B (obtained by Bio-Gel P-60 chromatography) and found no enhancement of protease binding to DNA at 0.6 M NaCl (Table II, experiment 3c). Even when less stringent binding conditions of 0.3 M NaCl are used (where 78% of the native or the reconstituted dimer input were

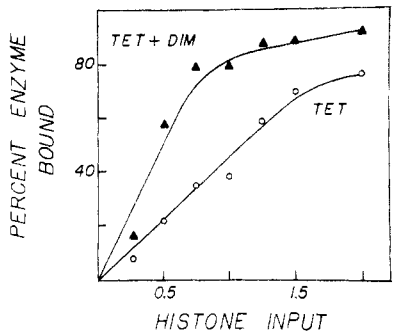

FIGURE 2: Effect of protein input upon subsequent binding of the protease to reconstituted DNA–protein complexes. The precentages of the protease bound to DNA–protein complexes (relative to the activity present in the unfractionated mixture) were examined as a function of increasing amounts of tetramer (TET) or octamer (TET + DIM) added. For comparison, the input of either tetramer or octamer is presented as the *percent* of the observed abundance of each component in native chromatin (for example, 100% octamer is an input of 0.8 mg of octamer/mg of DNA and 100% tetramer is an input of 0.4 mg of tetramer/mg of DNA).

found bound to DNA), the reconstituted dimer affords only marginal stimulation of protease binding (18% of input protease bound), unlike the "native dimer", which elicits 99% bound enzyme. By a number of other criteria, however, native and reconstituted dimers appear identical: they are cleaved by the H2A–protease at essentially equal rates, they are bound to DNA during reconstitution with similar efficiency, they are characterized by identical elution patterns from Sephadex G-100 columns, and they are equivalent in their ability to convert tetramer to octamer (results to be published). Further support to the conclusion that the enhancement of protease binding seen with "native dimer" preparations is due to the presence in these preparations of trace amounts of contaminating H3–H4 tetramer is derived from the following observations: (i) A pool of chromatographically isolated native dimer that has been repurified on the same column is significantly less efficient in promoting the binding of the protease to DNA, when compared to singly purified dimer. (ii) Direct addition of less than 5% tetramer (that is, adding 5% of the level of tetramer present in chromatin) to the reconstituted dimer, the protease, and DNA results in the successful reconstitution of the enzyme to the DNA (92% of input protease bound). (iii) Reconstituted dimer can also be made to facilitate protease binding by the addition of either isolated histone H3 or histone H4. Since the Bio-Gel P-60 purified fractions of H3 and H4 might be slightly cross-contaminated and because the functional subunit is the H3–H4 tetramer and not the H3 or H4 monomer, we believe the stimulation of protease binding observed with either H3 or H4 preparations is due to the small amount of reconstituted H3–H4 tetramer formed during the experiment.

The H2A–H2B Dimer Increases the Efficiency of Protease Reconstitution. From the above experiments, we have concluded that the presence of tetramer in a mixture of DNA, protease, and dimer is necessary in order to achieve efficient binding of the protease. The role of the H2A–H2B dimer during reconstitution could be indirectly determined by comparing the enhancement of protease binding by histone octamer to that elicited by H3–H4 tetramer. (Since all of our reconstitution experiments were conducted at pH 5.0, the "octamer" in the absence of DNA actually exists as a mixture of one H3–H4 tetramer and two H2A–H2B dimers. However, reconstitution of this material with DNA at pH 5 or 7.5 yielded "normal" nucleosomes, as will be documented in another publication.) Samples of constant amounts of DNA, protease,

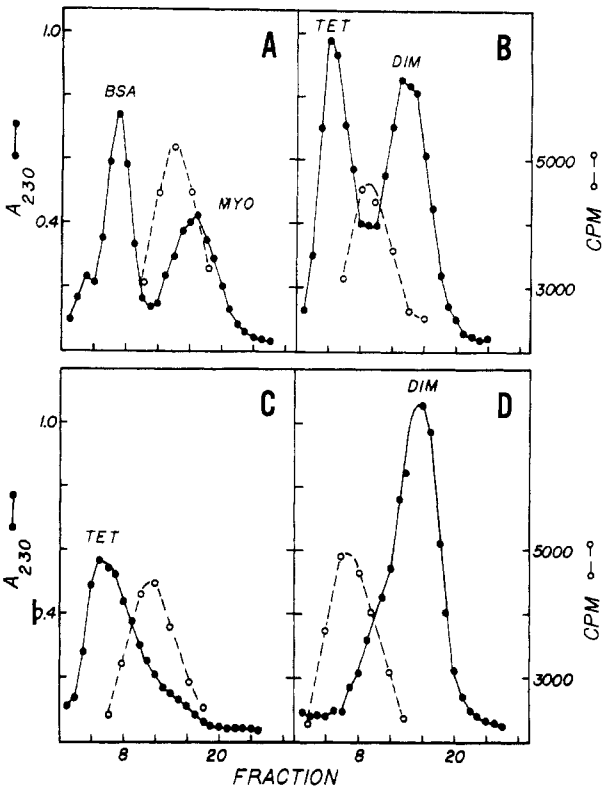

FIGURE 3: Sucrose gradient sedimentation of protease–protein mixtures. Sedimentation of samples on 5–20% linear sucrose gradients in 2.0 M NaCl and 10 mM NaH_2PO_4 (pH 5.5). Samples (400 μL) consisted of 100 μL of protease mixed with buffer and (A) 200 μL of BSA (fraction V, Sigma) and myoglobin (horse skeletal muscle myoglobin, type I, Sigma), each at 1 mg/mL, (B) 300 μL of the histone "octamer" at 3.3 mg/mL, (C) 200 μL of the H3–H4 tetramer at 2.3 mg/mL, or (D) 200 μL of the reconstituted dimer at 2.9 mg/mL. These mixtures were prepared and allowed to remain on ice for at least 1 h. Centrifugation was conducted in a Beckman SW50.1 rotor for 55 h at 4 °C and 42 000 rpm. Direction of migration was from right to left. Assays for protease were performed by incubation of 25 μL of selected gradient fractions with 175 μL of buffer (2.0 M NaCl and 10 mM glycine–NaOH, pH 9.0) and 50 μL of ^{3}H-labeled H2A (at 1 mg/mL) at 37 °C for 60 min. Protein was monitored by A_{230} of diluted fractions.

and H1 and increasing amounts of octamer or tetramer were prepared and dialyzed to low ionic strength. The efficiency of the binding of the protease to DNA as a function of increasing concentrations of tetramer or octamer is presented in Figure 2. The dimer can clearly effect protease binding when the tetramer is also present during complex formation. This can be compared to the inability of the dimer alone to aid protease binding when added to DNA (Table II). Mixing the reconstituted dimer with the H3–H4 tetramer prior to their addition to the reconstitution system also increases the amount of protease bound over that bound by tetramer alone. It is not likely that the binding enhancement seen with the octamer is due to the direct binding of the enzyme to its substrate, H2A, because although the reconstituted H2A–H2B dimer binds to DNA upon dialysis to 0.3 M NaCl, this DNA–dimer complex does not bind the protease efficiently (see above).

The Protease Forms a Stable Complex with the H2A–H2B Dimer but Not with the H3–H4 Tetramer. For determination of whether the protease can form a complex in solution with the tetramer, the dimer, and/or the octamer, sucrose gradient sedimentation of various mixtures was carried out, and the results are presented in Figure 3. From the position of the peak of protease activity in Panel A and panel D, it is clear that the protease is interacting strongly with the H2A–H2B dimer. Indeed, the shift in mobility to a position ahead of the

dimer peak is consistent with the formation of a stable complex between the protease and its substrate within the H2A–H2B dimer. When a mixture of the protease and the H3–H4 tetramer is run on the gradient (panel C), a small shift in mobility of the enzyme is also evident, but unlike the interaction shown with the dimer (panel D), the protease is not migrating ahead of the tetramer as would be expected if a tight complex had been formed. From its mobility on the gradient, it is likely that the tetramer aggregates (on these gradients, ovalbumin with molecular weight 43 000 sediments to fraction 9), and this aggregation may cause the enzyme to migrate into the gradient. In a separate experiment, where this aggregation was reduced (by loading less tetramer onto the gradient), the peak of the tetramer was found at fraction 10, and the protease was found at fraction 13 (the same position as that found on the gradient containing only marker proteins). These results indicate that the enzyme interacts very strongly with the H2A–H2B dimer but only weakly, if at all, with the H3–H4 tetramer. All of the gradients contained 2.0 M NaCl in order to (i) aid enzyme stability, (ii) minimize nonspecific interactions, and (iii) duplicate the conditions under which the octamer and its subunits have been characterized.

The Protease Can Be Bound to Preformed DNA–Protein Complexes. In the previous sections, we have demonstrated that the H3–H4 tetramer is required for the binding of the protease to artificial complexes. The order of protease and histone binding onto DNA during reconstitution is not clear; does the protease need to associate with the histones before they bind to the DNA, or can it bind to a preformed complex? For examination of this question, DNA–octamer complexes (prepared as described under Materials and Methods) were used as a model chromatin system. When the ionic strength is maintained at 0.6 M, the H2A–H2B dimer remains bound to the DNA along with the tetramer; we have observed that 0.9 M NaCl is sufficient to remove most of the dimer, while not affecting the binding of the H3–H4 tetramer to the DNA. After the addition of protease and histone H1 to the preformed DNA–octamer complexes, the buffer was adjusted to either 0.6 M NaCl or 0.9 M NaCl. The samples were subsequently dialyzed against 0.6 M NaCl, to allow the dimer to rebind to the DNA in the sample exposed to 0.9 M NaCl, and resolved by Bio-Gel A-50 chromatography. The material found in the excluded volume of the columns was assayed for bound enzyme. The preparations demonstrated only a slight difference in enzymatic activity (the 0.6 M NaCl pretreated sample had 85% of the activity found with the 0.9 M NaCl pretreated complex); thus, the protease is able to bind to the preformed DNA–octamer complex without requiring the removal and subsequent reassociation of the H2A–H2B dimer. Also because all of the input protease is bound without exposure of the DNA–octamer complex to ionic conditions high enough to remove measurable amounts of the H3–H4 tetramer, it appears that the protease is binding to the DNA after it has been compacted into a nucleosome-like structure by complexing with histone; it is not simply being trapped into the DNA–protein complex during its formation.

The Protease Is Found in Isolated Monomer Nucleosomes. We have examined the behavior of the protease during digestion of chromatin with micrococcal nuclease. Lysed calf thymus nuclei were extracted with 0.6 M NaCl and subsequently were used for isolation of H1-depleted chromatin (described under Materials and Methods). This chromatin was incubated with micrococcal nuclease, and the resultant digest was fractionated on a linear 5–20% sucrose gradient containing 0.4 M NaCl to minimize nonspecific ionic interactions between free protein and nucleosomes (Woodcock et al., 1976; Whitlock & Simpson, 1976). As can be seen (Figure 4), the majority of the chromatin has been digested to the size of monomer nucleosomes, and the small products of digestion are found near the top of the gradient. Pools from the monomer region and from the top of the gradient (indicated by the shaded areas) were each concentrated and repurified by gradient centrifugation to minimize their possible cross-contamination.

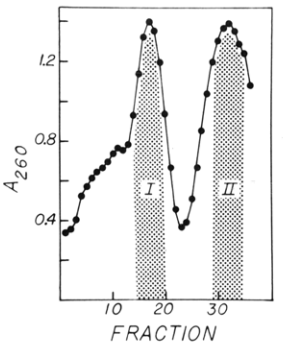

FIGURE 4: Isolation of the monomer pool and the released DNA fragments from a micrococcal nuclease digestion of 0.6 M NaCl extracted chromatin. Sucrose gradient centrifugation of the products from a 37% digestion of calf thymus chromatin. The direction of sedimentation is from right to left. Fractions from the total digest were pooled (as indicated), concentrated, and resedimented on a second gradient.

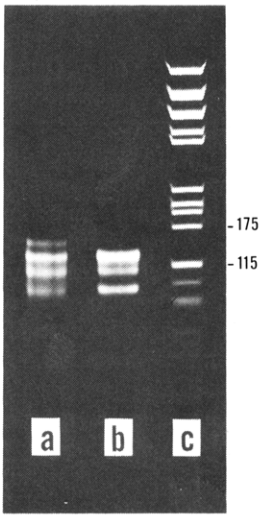

FIGURE 5: DNA from native and reconstituted chromatin digested with micrococcal nuclease. DNA from monomer preparations run on a 6% polyacrylamide gel. Channel a contains the DNA from the monomer nucleosome pool isolated from the chromatin digest presented in Figure 4. Channel b contains the DNA from the monomer nucleosome pool isolated from the reconstituted complex of octamer, protease, and DNA. Channel c contains DNA markers from an *Hae*III digestion of pMB9, provided by A. Efstratiadis [this preparation has fragment lengths of 800, 610, 540 (doublet of 560 and 500), 430, 400, 245, 220, 200, 175, 115, 100, 80–85, 65, 58 doublet, 42, and one smaller band].

The mobility of DNA purified from the monomer pool upon electrophoresis on polyacrylamide gels (Figure 5a) indicates that the digestion has removed the spacer DNA from the exterior of the core nucleosome. Indeed, digestion has been so extensive that the nuclease has cut the DNA within the 140-base-pair core.

The amount of protease present in a sample was assessed by the extent of H2A to cH2A conversion. For this determination, proteins isolated by 2.0 M NaCl extraction were incubated at 37 °C for various time periods and then examined

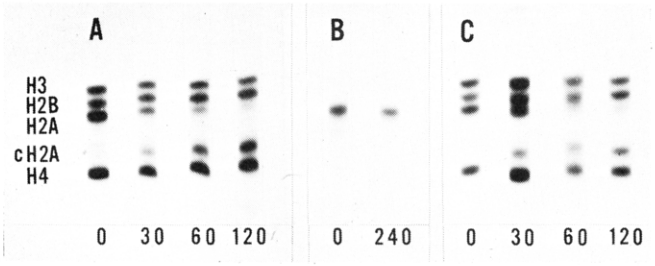

FIGURE 6: Protease activity of the pools from nuclease digestion of chromatin and control chromatin. Samples in 10 mM Tris and 1 mM EDTA (pH 8.0) were mixed with 4.0 M NaCl and 20 mM Tris-HCl (pH 9.0) to yield a final concentration of 0.25 mg/mL DNA in 2.0 M NaCl at pH 8.65. (A) Incubation of the isolated monomer pool for 0, 30, 60, and 120 min at 37 °C. Incubations of 30 and 60 min resulted in 42% and 79% cleavage, respectively. (B) Incubation of the isolated pool from the top of the gradient for 0 and 240 min. For this incubation, pure H2A was added before incubation to ensure comparable substrate concentration. (C) Incubation of control chromatin (0.6 M NaCl extracted chromatin) for 0, 30, 60, and 120 min at 37 °C. Incubations of 30 and 60 min resulted in 29% and 76% cleavage, respectively.

by acrylamide gel electrophoresis. As can be seen in Figure 6A, when the monomer nucleosome pool was incubated, complete cleavage of H2A occurred within 2 h. Thus, the protease is indeed isolated with monomer nucleosomes.

Several additional experiments were carried out to further characterize the distribution of the protease on chromatin. First, if the protease was also present on the internucleosomal DNA, the protein released during the nuclease digestion would demonstrate H2A-proteolytic activity. However, incubation of the material pooled from the top of the gradient (Figure 4, pool II, containing released fragments of DNA) with pure H2A for up to four hours did not result in any detectable cleavage of H2A (Figure 6B). Aliquots containing a known amount of purified protease and histone H2A were mixed with either buffer or the peak fraction from the top of the gradient to test whether this lack of proteolysis was due to some inhibitory factor being present in the material pooled from the top of the gradient. After 6 h of incubation, the extent of H2A cleavage in these mixtures were 23% and 22%, respectively. Thus, the inability to detect proteolytic activity in this fraction is not due to the presence of an inhibitor. Failure to detect protease activity within this pool may also be due to either an inactivation of the enzyme upon release from the DNA or pelleting of the released internucleosomal enzyme to the bottom of the gradient as a small DNA–protein complex due to charge neutralization with the DNA. Unfortunately, neither of these possibilities can be tested directly.

As an alternative approach, the amount of the protease associated with the H1-depleted chromatin before digestion was determined (Figure 6C), as described above for monomer nucleosome preparations, and found to be similar to that observed with the isolated nucleosomes. Thus, it can be concluded that most of the protease is found with the isolated nucleosomes and that there is no protease outside the monomer nucleosome pool.

For examination of the binding stability of the enzyme in isolated chromatin, soluble long chromatin fragments (oligonucleosomes were prepared as described under Materials and Methods) from calf thymus nuclei were mixed with an equal amount of monomer nucleosomes obtained from H1-depleted calf brain chromatin. Because the brain has much less proteolytic activity than thymus (Eickbush et al., 1976), a transfer of protease molecules between these preparations would be measurable. After the mixtures were incubated under the conditions used for the digestion of chromatin (37 °C for 60 min in the presence of 0.16 mM $CaCl_2$), the monomer nucleosomes and oligonucleosomes were separated on sucrose gradients (not shown) and assayed for proteolytic activity. No evidence for any transfer was detected; thus, the enzyme does not migrate between pieces of DNA during the isolation of the monomer nucleosomes used for these studies.

The Protease Is Found in Monomer Nucleosomes Isolated from Reconstituted Complexes. Using the ability of the protease to remain bound to chromatin in 0.6 M NaCl as a criterion for "proper" binding, we have described experiments showing that a mixture of the histone octamer, DNA, and the enzyme is adequate to allow effective reconstitution of the protease. Another criterion for proper DNA–protease reconstitution is that the enzyme should again be found in the monomer nucleosome pool obtained upon digestion of reconstituted complexes of DNA, the histone octamer, and the protease. Following micrococcal digestion of these reconstituted complexes, the monomer nucleosome pool and the low molecular weight pool were obtained as described for their isolation from chromatin. A sample of DNA from the monomer pool was electrophoresed to verify that the spacer DNA was absent from this preparation (Figure 5b). Two different protease assays were used to examine the amount of enzyme present in these pools and in the total complex before digestion. First, using the electrophoretic assay method described above, we find that the rate of appearance of cH2A is the same for the isolated monomer pool and for the undigested total complex. Incubation of the monomer nucleosome pool for 30 and 60 min resulted in 45% and 84% cleavage of H2A, respectively; comparative incubations of the total reconstituted DNA–protein complex resulted in 44% and 78% cleavage of H2A. Second, the radioactivity released from ^3H-labeled H2A during the 6-h incubation of the monomer pool is essentially identical with that released during the incubation of the original reconstituted complex (1811 and 2146 cpm, representing 444 and 453 cpm/A_{260}, respectively). No significant amount of activity can be detected with the pool obtained from the top of the gradient (16 cpm, representing 3 cpm/A_{260}). We conclude that the H2A–protease is found exclusively with monomer nucleosomes obtained from the digestion of reconstituted chromatin.

Discussion

The H2A-specific protease is the first nonhistone protein of defined activity whose binding to DNA is related to the binding of the histones to DNA. Early studies by others have provided qualitative evidence for interactions between histones and nonhistones in solution (Wang & Johns, 1968), while more recent experiments have described the relative strength of such interactions (Yu & Spring, 1977; McCleary et al., 1978) and have demonstrated a relationship between the binding of histone and nonhistone proteins to DNA (Gadski & Chae, 1976; Lepeyre & Bekhor, 1976).

In the present study, we have demonstrated that the protease is extracted from chromatin under conditions identical with those of the core histones. The observation that urea allows more of the protease to be released than that removed by the same ionic strength in the absence of urea demonstrates that forces other than ionic are operational in keeping the enzyme bound to chromatin. Although urea disrupts both hydrogen and hydrophobic interactions, studies on β-lactoglobulin have demonstrated that very high concentrations of urea (6.5 M) are required to disrupt hydrophobic bonds (Tanford & De, 1961). Since the protease was released from chromatin at low ionic strength with a relatively low concentration of urea, it is likely that the enzyme interacts with the chromatin com-

ponents by ionic and hydrogen bonds. That hydrogen bonding plays a significant role in the structure of chromatin by stabilization of the histone octamer has previously been proposed (Eickbush & Moudrianakis, 1978b).

We have determined which components of chromatin are required for the proper binding of the protease to DNA. DNA alone or even with trace amounts of adventitious proteins (unlikely in our DNA preparations) is not able to bind the protease. This is concluded from the observation that for the proper binding of the protease to our DNA preparation, specific types of histones must be added.

In light of the current models of chromatin structure, which depict structural changes in the DNA upon binding of the histones, it is not surprising that some nonhistone proteins would depend upon these histone-elicited conformational changes of the DNA for a proper binding environment. As a major structure of the chromatin is determined by the arginine-rich histones (Camerini-Otero et al., 1976; Boseley et al., 1976; Bina-Stein & Simpson, 1977), it is significant that the enzyme could be efficiently reconstituted to the DNA in the presence of only the H3–H4 tetramer, especially since we found that a direct interaction between the protease and this tetramer in solution is insignificant. On the other hand, the interaction between the enzyme and the H2A–H2B dimer is much stronger than that between the tetramer and the protease, yet the binding of the dimer alone to DNA at low ionic strength does not elicit proper protease reconstitution. It appears that something beyond protein–protein interaction is required for the proper association of the enzyme to DNA. We have concluded that the binding of the protease is dependent upon the structure of the DNA and is not dependent upon a direct interaction between the protease and its substrate, H2A, or with any of the other histones, although the histone dimer will enhance the level of reconstitution elicited by tetramer alone. A synergistic role for the H2A–H2B dimer in the overall structure of chromatin has been demonstrated. It has been shown that, although the H2A–H2B dimer alone did not offer any specific protection to DNA from the action of micrococcal nuclease, the H3–H4 tetramer did. However, the subsequent addition of dimer to a DNA–tetramer mixture increased the protection of the DNA and brought it to the level seen with chromatin digests (Camerini-Otero et al., 1976). Likewise, the ability of the dimer to add additional structure to DNA only in the presence of the tetramer has been shown by studies examining the effect of histone upon the induction of supercoils onto closed circular DNA (Camerini-Otero & Felsenfeld, 1977). By analogy, we have found that the H2A–H2B dimer is unable to elicit protease binding when present alone, but it clearly has a role in the binding of the enzyme when the H3–H4 tetramer is present. Thus, we propose that the binding of the protease to DNA requires the helix to assume a state analogous to that found in chromatin rather than the formation of a specific DNA–protein complex.

The H2A-specific protease is also the first nonhistone protein to be found associated *exclusively* in the core nucleosome and, when reconstituted to DNA in the presence of the histone octamer, binds in such a manner as to be recovered subsequently with the isolated monomer nucleosomes. Thus, in native chromatin and in chromatin-like complexes, the H2A–protease is in close proximity to its substrate. The majority of nonhistones whose distribution has been examined by nuclease digestion have been found to be internucleosomal (Mullins et al., 1977; Bohm et al., 1977). While some nonhistones have been reported in preparation of monomer nucleosomes isolated after brief digestion times (Goodwin et al., 1977; Schlaeger et al., 1978), only a few studies have found them associated with monomer nucleosome pools obtained after extensive digestion (for example, Chan & Liew, 1977).

There are two possible qualifications to the conclusion that all of the H2A–protease is associated with the core nucleosomes. First, no enrichment of the protease with the nucleosome preparation was observed: since all the comparisons are made assuming that no activation or inactivation of the enzyme occurs during digestion and fractionation procedures, a gradual loss of overall activity would diminish the expected increase in the specific activity of a true nucleosomal enzyme. Second, the ability of free enzyme to bind to preformed DNA–octamer complexes could result in an overestimate of the amount of enzyme associated with the nucleosome. Thus, although the protease is found with isolated nucleosomes, the determination of the distribution of the enzyme will have to be verified by an alternate method, such as electron microscopy of native chromatin and nuclei previously stained with labeled antibodies directed against the protease. We recognize that accessibility problems may preclude the use of antibodies for the identification of the protease in situ and may necessitate the use of a more diffusable, but less specific, ligand, such as DFP (diisopropyl fluorophosphate), for this identification.

What could be the purpose of an enzyme placed within the nucleosome structure? Analysis of the amount of enzyme bound at a low octamer/DNA input ratio (Figure 2), as compared to that found with isolated calf thymus chromatin, allows us to estimate that the protease is associated with less than 5% of the nucleosomes (assuming one enzyme site per octamer). Perhaps the cell is retaining the enzyme in a sequestered form until it may be required. It is known that the H2A–protease is able to either open the octamer or shift the equilibrium from an octamer toward a H3–H4 tetramer and cH2A–H2B dimers in solution (T. H. Eickbush and E. N. Moudrianakis, unpublished experiments); thus, the enzyme may be able to completely destabilize the nucleosome in vivo. It has been shown that enzymes are able to recognize chromatin that has been structurally modified, perhaps due to altered accessibility. For example, acetylation of chromatin changes the ability of RNA polymerase to transcribe it (Marushige, 1976) and changes the rate at which chromatin is digested by nucleases (Wallace et al., 1977; Simpson, 1978; Vidali et al., 1978). Similar chromatin modifications may allow a sequestered enzyme to work, resulting in changes in chromatin structure. Experiments to investigate these possibilities are currently underway.

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