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Differential Accessibility of the Amino and Carboxy Termini of Histone H2A in the Nucleosome and Its Histone Subunits†

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ABSTRACT: In our efforts to determine the role of the contact interface of the H2A:H2B dimer and the $(H3:H4)_2$ tetramer as a possible site for the regulation of nucleosome structure and function, we have probed this interface by the use of proteolytic enzymes. The accessibility of the amino and carboxy termini of H2A to cleavage by trypsin was tested in the H2A:H2B dimer, the core histone octamer, chromatin, and nuclei. The H2A-specific protease [Eickbush, T. H., Watson, D. K., & Moudrianakis, E. N. (1976) *Cell* (*Cambridge, Mass.*) *9*, 785–792] was utilized to establish the order of cleavage of the terminal regions of H2A. Three consecutive slab gel electrophoretic systems were used to monitor the histone 2A cleavage products. In the absence of DNA, the amino-terminal region of H2A was observed to be readily cleaved by trypsin when the H2A:H2B dimer is associated with the $(H3:H4)_2$ tetramer in the form of a solubilized octamer. However, in this case, the carboxy terminus of the molecule is quite resistant to cleavage by trypsin. In contrast, when the substrate for trypsinization was the H2A:H2B dimer or several forms of chromatin or nuclei, there was an ordered cleavage of the amino terminus of H2A followed by cleavage of its carboxy-terminal region. It therefore appears then that the carboxy terminus of H2A is somehow involved in the formation of the dimer–tetramer contact interface in the octamer. Furthermore, this interface is accessible to external enzymatic modifications when the octamer is associated with DNA in the form of a nucleosome, while it appears to be much less accessible in free octamers as they exist in high ionic strength solutions. This observation may have important implications in the regulation of chromatin structure through modification of specific regions along this contact interface by either covalent or associative interactions, both of which could alter the compaction state of the nucleosome.

In recent years, a great deal of experimental data has been gathered on the organization of histones and DNA in chromatin. Brief digestion of eukaryotic chromatin with micrococcal nuclease yields particles call nucleosomes which contain about 200 base pairs of DNA associated with two each of the four core histones H2A, H2B, H3, and H4 in the form of histone octamer, and one molecule of H1. Further nuclease digestion brings about the production of the core nucleosome particle which consists of 146 base pairs of DNA wrapped around the core histone octamer [see review by McGhee & Felsenfeld (1980)]. The octamer, which is extractable as a unit from chromatin in 2 M NaCl, is formed by the association of two H2A:H2B dimers with an $(H3:H4)_2$ tetramer (Eickbush & Moudrianakis, 1978). This laboratory has previously presented the results of gel filtration and sedimentation equilibrium experiments which indicate that in solutions of high ionic strength, the core histones behave as a reversibly associating system of histone octamers in equilibrium with $(H3:H4)_2$-H2A:H2B hexamers, $(H3:H4)_2$ tetramers, and H2A:H2B dimers (Godfrey et al., 1980). The balance of this equilibrium is affected by the ionic strength, pH, temperature, and the presence of urea. On the basis of these findings, it has been suggested that the interaction of the dimer and the tetramer involved predominately the formation of a limited number of hydrogen bonds (Eickbush & Moudrianakis, 1978). It is our hypothesis that the metastable dimer–tetramer contact interface may function as a site of regulation of nucleosome function.

A great amount of work has been done to establish the histone–histone contacts within the nucleosome. Much of this has been accomplished through the use of chemical cross-linking reagents. Another approach has involved the use of proteolytic enzymes to determine the accessibility of the histones to their external environment. Weintraub & Van Lente (1974) also examined the digestion of the core histones when they are released from the DNA in 2 M NaCl. Trypsin-modified nucleoprotein complexes were subjected to DNase I digestion (Whitlock & Simpson, 1977) and protein cross-linking with dimethyl suberimidate (Whitlock & Stein, 1978), and it was concluded that the trypsin-limit peptides of the core histones retained both the structural elements necessary for proper protein–protein associations and the ability to organize the DNA into a nucleoprotein complex which resembled the chromatin core particle. A series of recent studies by Bohm and co-workers (Bohm et al., 1980, 1981, 1982) have defined the limit digest products of the core histones when chicken erythrocyte nuclei were extensively digested with trypsin. Of particular interest to this study was the characterization of the major trypsin-limit peptide of H2A which was comprised of residues 12–118 from the original H2A of 128 amino acids in length.

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In the present study, we have utilized a combination of high-resolution gel electrophoretic techniques to assay the accessibility of the amino- and carboxy-terminal regions of H2A to trypsin when organized in a number of relevant chromatin structures, i.e., the H2A:H2B dimer, the core histone octamer, and several nucleoprotein structures. The H2A-specific protease was also used to help establish the order of cleavage of the terminal regions of H2A in the dimer and octamer. Eickbush et al. (1976) found that under defined conditions this protease recognizes and cleaves a specific site on the H2A molecule between valine$_{114}$ and leucine$_{115}$. We have observed that although the amino-terminal region of H2A is cleaved by trypsin when the H2A:H2B dimer is associated with the $(H3:H4)_2$ tetramer as in the octamer, the carboxy terminus of the molecule is quite resistant to cleavage by trypsin. In contrast, when the substrate for trypsinization is the H2A:H2B dimer alone or several forms of chromatin or nuclei, there is an ordered cleavage of the amino terminus of H2A followed by cleavage of its carboxy-terminal region. The reproducible and ordered production of a limited number of distinct electrophoretic bands indicates the existence of specific and differential barriers to the accessibility of the amino and carboxy termini of H2A to cleavage by trypsin. Furthermore, the cleavage patterns of H2A in the various substrates tested indicate that the dimer and tetramer exhibit a different, and perhaps tighter, association when they assemble as an octamer in high ionic strength solutions than when they are assembled with DNA to form the nucleosome.

Materials and Methods

Protein Purification. Core histone octamer and H2A:H2B dimer were purified as previously described (Eickbush & Moudrianakis, 1978). Protein concentrations were determined by the biuret protein assay as described by Zamenhoff (1957).

Proteolytic Treatments of the H2A:H2B Dimer. H2A:H2B dimer was column purified as in Eickbush & Moudrianakis (1978) and vacuum concentrated to approximately 4 mg/mL.

Native dimer at 0.5 mg/mL in 2 M NaCl, pH 7.5, was treated with 5 μg/mL trypsin (from Worthington Biochemical Corp.; TPCK[1] pretreated) at 4 °C for 7.15, 15, 45, and 135 min. Equal aliquots were removed at each time point, treated with 2 mM PMSF, and processed for gel electrophoresis.

Native dimer, predialyzed to 2 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 8.0–8.2, was treated with the H2A-specific protease for 90 min at 37 °C to bring about a 50% conversion of the native form of H2A to a form which has lost 15 carboxy-terminal amino acids (Eickbush et al., 1976). At the end of this incubation, the sample was placed on ice. One-fifth of the mixture was immediately desalted and lyophilized. Another fifth of the mixture was left on ice for 135 min before being desalted and lyophilized. The remainder received trypsin at 5 μg/mL and was digested for 15, 45, and 135 min. At each time, one-fifth of the original volume was removed, treated with 1 mM PMSF, desalted, and lyophilized.

Proteolytic Treatments of the Core Histone Octamer. In the first procedure, octamer in 2 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5, at concentrations from 4.0 to 18.0 mg/mL was digested with TPCK–trypsin (prepared and donated by Sylvia Himmelfarb in the laboratory of Dr. W. F. Harrington) at 5–34 μg/mL until the more trypsin-resistant histone core was produced as monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown). Soybean trypsin inhibitor (SBTI) was added in a 15–20 times weight excess over trypsin, and the incubation mixture was chromatographed through a G100 column in 2 M NaCl–10 mM Tris, pH 7.5. This material migrated as a single peak and was found to contain all four histone-limit peptides (data not shown). The material from this peak was pooled and vacuum concentrated to 1–3 mg/mL. Aliquots of this trypsin form of octamer were then treated with H2A-specific protease at 37 °C in the same solvent but at pH 8.0–8.2. Control samples were incubated in parallel without added protease. No contaminating proteolytic activity was observed.

In an alternative procedure, 0.5–14.0 mg/mL octamer in 2 M NaCl, pH 7.5, was digested with TPCK–trypsin until the limit peptides were produced. The action of trypsin was halted by the addition of fresh 100 mM PMSF in 2-propanol in 0.5 mM steps until a concentration of 2 mM was reached. The repeated addition of fresh PMSF was critical to the inhibition of further trypsin activity. An aliquot was saved for electrophoretic analysis. The bulk of the material was dialyzed overnight vs. several changes of 2 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 8.2. These samples were divided into two parallel samples which were incubated at 37 °C in the presence or absence of added H2A-specific protease. Electrophoretic analysis showed that there was no further degradation in the absence of added protease (data not shown).

Proteolytic Treatment of Partially Dissociated Octamer. Octamer at 0.5 mg/mL in 0.6 and 1.2 M NaCl with 10 mM Tris–1 mM EDTA, pH 7.5, was digested with TPCK–trypsin at 5 μg/mL. At given times, aliquots were removed, treated with 2 mM PMSF, and processed for gel electrophoresis.

Trypsin Digestion of Nuclei and Chromatin. Calf thymus tissue (stored at −80 °C until use) was homogenized, filtered through preboiled cheesecloth and Miracloth, and washed in the presence of 0.2 M sucrose, 5 mM Hepes, 3 mM $MgCl_2$, and 0.6 mM $CaCl_2$, pH 7.3 (buffer A), plus freshly added 1 mM PMSF. The nuclei were washed, spun (2500 rpm, 10 min, SS-34 rotor), and resuspended in this buffer for at least three cycles. Aliquots of nuclei in this buffer were removed and utilized directly for trypsinization. Nuclei at a concentration of 0.5–3.0 mg/mL acid-extractable protein were digested for varying lengths of time with 3–20 μg/mL TPCK–trypsin. The remainder of the nuclei were used to make two types of chromatin. H1-depleted long soluble chromatin was isolated by the procedure of Lutter (1978) with the following modification: the A5m column was eluted in the presence of 0.5 M NaCl rather than 0.45 M NaCl. Some of the nuclei which had been washed at least 3 times in buffer A plus PMSF were washed twice with 0.14 M NaCl, 10 mM Tris, 3 mM $MgCl_2$, and 0.6 mM $CaCl_2$, pH 7.5, and then twice with 0.35 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5. Both buffers received fresh 1 mM PMSF after resuspension of the nuclear material. The final pellets were swollen in deionized water for several hours and then spun down in the presence of 4 mM EDTA, pH 7.5 (5000 rpm, 15 min, GSA rotor). This process was repeated once more. The final pellets were then collected and mildly sheared in a Waring Blendor, and the A_{260nm} values were determined. Aliquots of this chromatin (A_{260nm} = 25) were brought to the desired ionic strength by addition of an equal volume of a 2× salt plus buffer solution and then incubated for varying lengths of time with TPCK–trypsin at 10–20 μg/mL. H1-depleted long soluble chromatin purified through the A5m column was either left in the 0.5

[1] Abbreviations: EDTA, disodium (ethylenedinitrilo)tetraacetic acid (from Baker); $NaDodSO_4$, sodium dodecyl sulfate (from Sigma); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (from Sigma); PMSF, phenylmethanesulfonyl fluoride (from Sigma); TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (from Cyclo Chemical Corp.); Tris, tris(hydroxymethyl)aminomethane.

M NaCl buffer of the column or was dialyzed to 0.25 mM EDTA, pH 7.5, before being incubated with TPCK–trypsin for varying lengths of time at 4 °C.

Chick erythrocyte nuclei which had been extensively washed in 10 mM NaCl, 3 mM $MgCl_2$, and 10 mM Tris, pH 7.4 (STM buffer), were diluted 1:1 with glyerol and stored at −80 °C until use (Olins et al., 1976). The nuclei were thawed and washed 3 times with 0.3 M sucrose–STM buffer plus freshly added 0.5 mM PMSF. Nuclei at approximately 200–500 μg of acid-extractable protein per mL were digested with 2–5 μg/mL TPCK–trypsin for increasing lengths of time. Unless indicated otherwise, all manipulations of the nuclei and chromatin were done at 4 °C.

Proteolytic Treatments of H2A:H2B Dimer and Core Histone Octamer after Reconstitution to Sheared Calf Thymus DNA. Calf thymus H2A:H2B dimer and core histone octamer were reconstituted separately to sheared calf thymus DNA by salt gradient dialysis. The protein and DNA were mixed at a weight/weight ratio of 0.8 in 2.0 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5. The NaCl concentration was reduced stepwise to 1.2, 0.9, 0.6, and 0.3 M and finally to the Tris–EDTA buffer alone. Each step lasted for 5 h or longer at 4 °C. The step at 0.6 M was done overnight, and the final step was done several times. Reconstitutes (at a DNA concentration of approximately 0.3 mg/mL) were treated with 40 μg/mL TPCK–trypsin at 4 °C. Aliquots of 175 μL were removed at 5, 25, and 100 min, and the proteolysis was stopped by the addition of 4 μL of 200 mM PMSF in ethanol. After 5 min, the samples were made 0.6 N HCl by the addition of 20 μL of 6 N HCl. Twenty minutes later, the DNA precipitate was pelleted by a 1-min centrifugation in a Beckman microfuge. Finally, the supernatant received 150 mg of urea, 20 μL of glacial acetic acid, 10 μL of 30% NH_4OH, and 2.5 μL of 2-mercaptoethanol. Each mixture was then directly loaded onto an AUT gel, and the histones were analyzed by the AUT–AUC two-dimension gel electrophoretic system described below.

Preparation of Proteins for Electrophoresis. Protein samples were collected after appropriate treatments, desalted over small columns of Sephadex G25 coarse run in 50 mM acetic acid, and then lyophilized to dryness. Dry samples were stored at −20 °C until preparation for gel electrophoresis. Histones were extracted from nuclei and chromatin with 0.5 N HCl at 4 °C for 15–30 min. The DNA was pelleted at 7500 rpm for 10 min in an SS-34 rotor, and the supernatants were desalted and lyophilized as above.

Electrophoretic Techniques. Three consecutive slab gel electrophoretic systems were utilized. The first dimension was the acid–urea–Triton X-100 (AUT) system of Bonner et al. (1980) as modified from Alfageme et al. (1974). The second dimension was the acid–urea–cetyltrimethylammonium bromide (AUC) system of Bonner et al. (1980). Individual protein spots from the AUC gels were electrophoresed in the $NaDodSO_4$–polyacrylamide system of Laemmli (1970) as modified by Thomas & Kornberg (1975), except that 375 mM Tris replaced 750 mM Tris in the resolving gel. This $NaDodSO_4$ system was also used directly for the analysis of the progress of proteolytic digestion of the various substrates being tested.

Results

In this study, we have focused on the proteolytic cleavage of the predominant H2A variant, H2A.1, which represents approximately 70% of the total H2A. In order to facilitate discussion of this work, we have adopted the following terminology for native H2A and the various classes of H2A

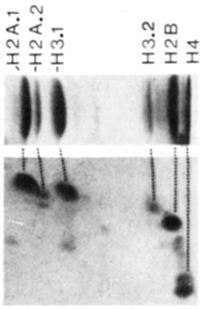


FIGURE 1: Core histone separation in one- and two-dimensional gel electrophoresis. The horizontal gel lane at the top of this figure shows the electrophoretic separation (from left to right) of the core histones of the octamer in the AUT first-dimension gel. This lane was placed onto an AUC second-dimension gel, and the proteins were electrophoresed directly into this gel. The dotted lines indicate the final positions of each of the major core histone variants in the AUC gel. In subsequent figures, only the vertical region of the AUC gel which contains H2A.1 and H2A.2 is shown.

cleavage products. Each is labeled as "H2A" followed by a two-part subscript. The first subscript refers to the amino terminus of the molecule, and the second subscript refers to the carboxy terminus of the molecule. The subscripts are defined as follows: n, native; t, trypsin cleaved; p, H2A-specific protease cleaved. The following examples illustrate this terminology: $H2A_{n,n}$ is the native form of H2A.1, $H2A_{t,n}$ indicates an H2A.1 which has lost some of its amino-terminal region by the action of trypsin but retains an intact carboxy terminus, and $H2A_{n,p}$ indicates the form of H2A.1 which has a native amino terminus but has lost the 15 carboxy-terminal amino acids as the result of treatment with the H2A-specific protease [$H2A_{n,p}$ is equivalent to the term cH2A used in Eickbush et al. (1976) and Watson & Moudrianakis (1982).

Three consecutive slab gel electrophoretic systems were used to monitor these histone 2A cleavage products resulting from the controlled proteolysis of a number of relevant chromatin structures. In the first electrophoretic dimension, the five histone classes, including both the individual class variants and the products of their cleavage, were partially resolved in the AUT gel system of Bonner et al. (1980). The migration of all the H2A forms is very similar in AUT, presumably because the Triton X-100 binding of the H2A core is not greatly altered by removing the termini. The AUT dimension in essence serves to separate the H2A peptides from the other histones (see Figure 1). Individual lanes or sections of lanes containing the H2A peptides of interest were electrophoresed for the second dimension in AUC gels which resolved the different H2A peptides. For this reason, the pattern of H2A cleavage observed on AUC gels can be exhibited as a montage of photographs of just the vertical region of the gel(s) containing native H2A.1 or its cleaved products.

The simplest associated system studied was the purified H2A:H2B dimer. When the dimer was treated with trypsin in 2 M NaCl, pH 7.5–8.0, $H2A_{n,n}$ was first converted to $H2A_{t,n}$ and then progressively to $H2A_{t,t}$ (Figure 2a–e). By 7.5 min, all the $H2A_{n,n}$ had been converted to $H2A_{t,n}$, and some had been converted to $H2A_{t,t}$. The cleavage on the C-terminal side of the molecule was detected at a later time, being almost complete at 135 min.

Trypsin and the H2A-specific protease cleave the C-terminal end of H2A in different positions. The innermost site for trypsin cleavage is likely to be at Lys_{118} as found by Bohm et al. (1980), while for the other enzyme the cleavage site is between Val_{114} and Leu_{115}. One would expect then that $H2A_{n,n}$ which was treated first with the specific protease to generate

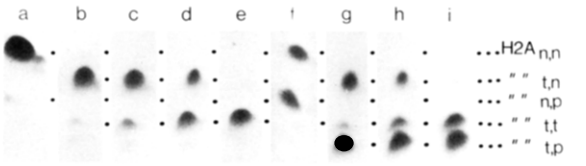
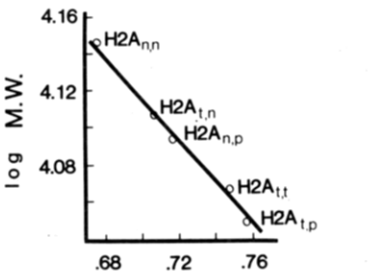


FIGURE 2: Montaged presentation of individual H2A regions of second-dimension AUC gels after treatment of the dimer with trypsin and the H2A-specific protease. (a) Native H2A.1 ($H2A_{n,n}$) from purified H2A:H2B dimer. (b–e) H2A.1 cleavage products resulting from incubation of native H2A:H2B dimer with trypsin for 7.5, 15, 45, and 135 min. (f) H2A.1 products resulting from treatment of native H2A:H2B dimer with the H2A-specific protease until 50% of the $H2A_{n,n}$ was converted to $H2A_{n,p}$. (g–i) H2A.1 cleavage products resulting from treatment of the material in (f) with trypsin for 15, 45, and 135 min.

$H2A_{n,p}$ and subsequently treated with trypsin would generate a product, $H2A_{t,p}$, which should be slightly smaller than $H2A_{t,t}$. Native dimer was cleaved to a 50% level with the H2A-specific protease as indicated by the appearance of an equal amount of stained material at the position of $H2A_{n,n}$ and $H2A_{n,p}$ on an AUC gel (Figure 2f). The protease cleavage was terminated by cooling the incubation mixture to 4 °C. Trypsin was added, and the mixture was digested for increasing amounts of time. Two concomitant cleavage patterns were observed. The $H2A_{n,n}$ was cleaved to $H2A_{t,n}$ which was in turn cleaved to the end product, $H2A_{t,t}$. The $H2A_{n,p}$ was quickly cleaved to its trypsin-resistant peptide, $H2A_{t,p}$ (Figure 2f–i).

For further establishment of the identity of each of these H2A species, the bands were excised from AUC gels and electrophoresed on $NaDodSO_4$–polyacylamide gels. The Coomassie Blue dye front was used to establish R_f values. It is known that the relative electrophoretic mobility of the various histones in $NaDodSO_4$–polyacrylamide gels is not linearly related to the log of their molecular weights (Panyim & Chalkley, 1971). This anomaly could be a reflection of the relative abundance of the hydrophobic and polar residues between the different histone polypeptides. However, in this study, we are comparing the mobility of the intact H2A polypeptide to its major cleaved product forms. We use two known fragments of the H2A molecule as size references, i.e., the limit trypsin-digest product of H2A established by Bohm et al. (1980) and the large-size fragment of H2A (cH2A or $H2A_{n,p}$) produced by the action of the H2A-specific protease (Eickbush et al., 1976) as well as the intact H2A molecule. In this tightly controlled system, a linear relationship can be obtained between the log of the molecular weight of the various H2A polypeptide forms and their relative mobilities in $NaDodSO_4$–polyacrylamide gels (Figure 3). Thus, we can estimate the most likely cleavage sites. Of course, the absolute identification of these sites would require the determination of the primary structures of these peptides or at least fingerprint analysis of their tryptic peptides. Nevertheless, our method of analysis indicates that the most probable inner sites of trypsin cleavage for H2A in this study are those defined by Bohm et al. (1980).

The next phase of the study was to trypsin treat core histone octamer in 2 M NaCl, pH 7.5, 4 °C, until the limit peptides of each of the core histones were produced. A single dimension of $NaDodSO_4$–polyacrylamide gel electrophoresis was used to monitor the progress of trypsin digestion as well as to allow comparison of the mobilities of these core histone cleaved products with those observed in a series of previously published studies (Weintraub & Van Lente, 1974; Weintraub, 1975; Whitlock & Simpson, 1977). We were able to identify the same core histone trypsin-cleaved products (termed P1–P5) originally identified by Weintraub & Van Lente (1974). This



	Rf	log M.W.	# A. Acids
$H2A_{n,n}$	.675	4.15	129
$H2A_{t,n}$	.705	4.11+	118
$H2A_{n,p}$	.717	4.09	114
$H2A_{t,t}$	.748	4.07+	107
$H2A_{t,p}$	.758	4.05+	103

+ estimated

FIGURE 3: Specific cleavage products of H2A.1. Dimer, octamer, chromatin, and nuclei were subjected to various proteolytic treatments and electrophoresed in the two-dimensional AUT–AUC gel system. Individual H2A.1 cleavage products were isolated from the AUC gels and electrophoresed in high-resolution $NaDodSO_4$–polyacrylamide gels. The R_f values were determined relative to the free Coomassie Blue dye front. For each type of cleavage product, the R_f values were very similar irrespective of the starting material, and therefore, each R_f value is an average of the electrophoresis of many spots from AUC gels. The plot of R_f vs. log molecular weight is shown. The table gives the numerical values of R_f, log molecular weight, and number of amino acids in each type of cleavage product. The right-hand superscript with some of the numbers indicates those values which were estimated from literature cited in the text.

allowed us to conclude that our core histone preparation had been trypsinized as thoroughly as in these previous studies. All of the trypsin-cleaved forms of the core histones were found to chromatograph as a single peak of "octamer" through Sephadex G100 eluted with 2 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5 (data not shown).

Using the two-dimension AUT–AUC gel system, we were able to show that in the H2A:H2B dimer both the N and C termini of H2A are available for trypsin cleavage and that the C terminus is available for cleavage by the H2A-specific protease. However, when complexed in an octamer, AUT–AUC gel analysis showed that the accessibility of the C terminus of H2A was altered. Core histone octamer in 2 M NaCl, pH 7.5, 4 °C, was digested with trypsin until all the limit peptides of all the core histones were produced. The native form of H2A.1, $H2A_{n,n}$ (Figure 4a), was found to be converted to a single faster migrating form, $H2A_{t,n}$, on AUC and $NaDodSO_4$ gels (Figure 4b and Figure 3, respectively). The cleavage pattern was the same over a wide variety of protein concentrations, 0.5–18.0 mg/mL (data not shown). Note that $H2A_{t,t}$ was absent, even though the cleavage reaction for both octamer and dimer was carried out in comparable conditions. The H2A C terminus seems to be protected in some way by its association with the $(H3:H4)_2$ tetramer.

This material was further treated with the H2A-specific protease to substantiate that the product of trypsin cleavage of H2A in the octamer was $H2A_{t,n}$. Elevation of the temperature from 4 to 37 °C causes the octamer to dissociate into its component dimers and tetramers (Eickbush & Moudrianakis, 1978) and allows rapid cleavage of native H2A by the H2A-specific protease (Eickbush et al., 1976). A distinct product, $H2A_{n,p}$, of increased mobility on AUC and $NaDodSO_4$–polyacrylamide gels was produced (Figure 4e,f; Figure 3). Similarly, the $H2A_{t,n}$ species produced by trypsinization

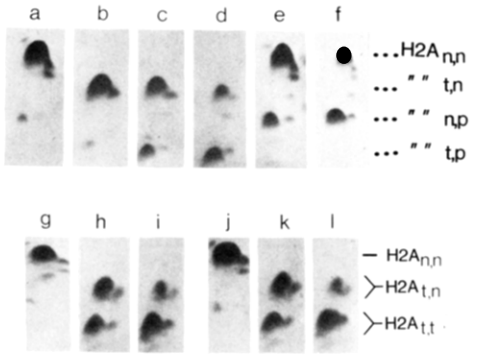


FIGURE 4: H2A.1 polypeptides which result from the treatment of core histone octamer with trypsin and the H2A-specific protease in 2 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.5–8.0. (a) $H2A_{n,n}$, control native H2A.1 from octamer. (b) H2A.1 peptide ($H2A_{t,n}$) which results from trypsinization of octamer in 2 M NaCl, pH 7.5. (c and d) H2A.1 peptide(s) resulting from 20 or 60 min of treatment of trypsinized octamer [as in (b)] with the H2A-specific protease. (e and f) H2A.1 peptide(s) resulting from 20 or 60 min of treatment of native octamer with the H2A-specific protease. Note that there is a small amount of $H2A_{n,p}$ in (a). This is the result of a small amount of endogenous H2A-specific protease activity during the purification of the octamer. In a parallel set of experiments, core histone octamer was trypsin treated in 0.6 and 1.2 M NaCl, pH 7.5, and the resulting H2A.1 products were analyzed. (g) $H2A_{n,n}$, control native H2A.1 from octamer in 0.6 M NaCl, pH 7.5. (h and i) H2A.1 cleavage products resulting from trypsinization of octamer (0.5 mg/mL) in 0.6 M NaCl, pH 7.5, for 30 or 90 min, respectively. (j) $H2A_{n,n}$, control native H2A.1 from octamer in 1.2 M NaCl, pH 7.5. (k and l) H2A.1 cleavage products resulting from trypsinization of octamer (0.5 mg/mL) in 1.2 M NaCl, pH 7.5, for 30 or 90 min, respectively.

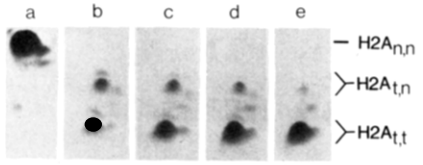


FIGURE 5: H2A.1 polypeptides resulting from the trypsinization of calf thymus nuclei. (a) $H2A_{n,n}$, control native H2A.1 from untrypsinized calf thymus nuclei. (b, c, d, and e) H2A.1 cleavage products resulting from trypsinization of calf thymus nuclei for 0.75, 2.25, 7, and 21 h, respectively.

of octamer in 2 M NaCl, pH 7.5, 4 °C, was cleaved to a faster migrating band, $H2A_{t,p}$, on AUC and $NaDodSO_4$ gels (Figure 4b–d; Figure 3). This form, $H2A_{t,p}$, had an identical electrophoretic mobility whether produced by the trypsinization of octamer followed by H2A-specific protease treatment ($H2A_{n,n} > H2A_{t,n} > H2A_{t,p}$) or by H2A-specific protease treatment of the native dimer followed by the trypsinization of this form of dimer ($H2A_{n,n} > H2A_{n,p} > H2A_{t,p}$). Note that the $H2A_{t,t}$ produced by trypsinization of dimer is of a slightly slower mobility than $H2A_{t,p}$ on both AUC and $NaDodSO_4$ gels (Figures 2 and 3). This suggests that this trypsin cleavage event may occur at the closest susceptible site from the specific protease cleavage site and toward the C terminus, i.e., between residues 118 and 119.

The dimer need not be purified to effect the pattern of cleavage previously described. Trypsinization of a 0.5 mg/mL solution of octamer in 0.6 or 1.2 M NaCl, pH 7.5, results in the same cleavage pattern (Figure 4g–l) as found for dimer alone, since these conditions enhance the dissociation of the octamer into its subunits. Indeed, because the histone octamer is in a readily reversible equilibrium with its subunits, it is a natural consequence that even in 2 M NaCl, pH 7.5, this pattern of cleavage by trypsin will eventually be observed (data not shown).

When calf thymus nuclei were trypsinized, the pattern of H2A cleavage was similar to that observed when free dimer was trypsinized. $H2A_{n,n}$ was initially converted to $H2A_{t,n}$ primarily and to $H2A_{t,t}$. With increasing time, all of the $H2A_{t,n}$ was converted to $H2A_{t,t}$ (Figure 5). Some heterogeneity in the $H2A_{t,n}$ and $H2A_{t,t}$ peptides is visible.

Mildly sheared, 0.35 M NaCl preextracted calf thymus chromatin was digested with trypsin in ionic strengths of 0, 0.15, and 0.5 M NaCl (Figure 6). Proteolysis was fastest in 0.5 M NaCl. It was approximately 3 times slower in 0.15 M NaCl and another 3 times slower in the absence of NaCl. In all of these conditions, three forms of $H2A_{t,n}$ were produced. With increasing time, these three forms are converted to faster mobility $H2A_{t,t}$ forms.

H1-depleted long soluble chromatin was digested with trypsin in low (no NaCl) and high (0.5 M NaCl) ionic strength buffers. In 0.5 M NaCl, the $H2A_{n,n}$ of this substrate was rapidly cleaved to produce $H2A_{t,n}$, and this in turn was cleaved to several forms of $H2A_{t,t}$ (data not shown). Although the same general pattern of cleavage was observed in low ionic strength, the rate of cleavage was greater than 100-fold slower.

It was observed that $H2A_{t,n}$ and $H2A_{t,t}$ were composed of a single peptide each when the trypsin digestion of free H2A:H2B dimer in solution was examined. However, when the digestion of chromatin was analyzed, these two classes of cleaved H2A forms exhibited three major peptides each. In order to understand the origin of these peptides, we reconstituted purified H2A:H2B dimer and core histone octamer separately with sheared calf thymus DNA. When these histone–DNA complexes were treated with trypsin, the same complexity in the pattern of $H2A_{t,n}$ and $H2A_{t,t}$ peptides was observed as when chromatin was digested with trypsin. The tryptic peptides of H2A produced during the trypsinization of H2A:H2B dimer–DNA complexes are shown in Figure 7.

In these experiments, the calf thymus nuclei and chromatin had been treated thoroughly with PMSF during preparation to eliminate the activity of possible contaminating endogenous serine proteases. As a further control, we analyzed the trypsin digestion of chick erythrocyte nuclei, which is a substrate with very low endogenous proteolytic activity and no detectable H2A-specific protease. Trypsinization gave rise to three forms

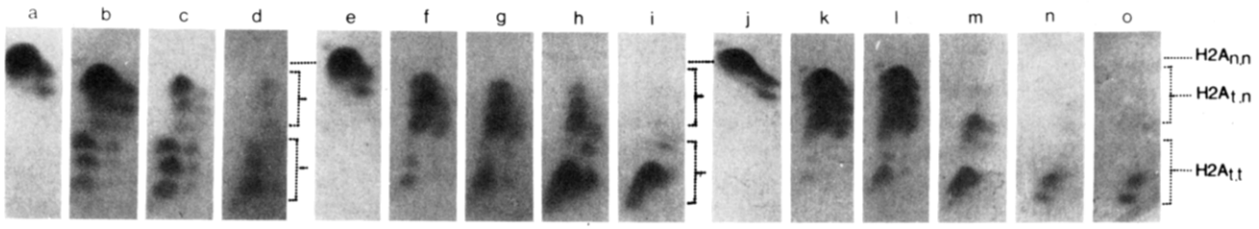


FIGURE 6: H2A.1 and its cleaved forms which result from the trypsinization of 0.35 M NaCl preextracted calf thymus chromatin in 0.25 mM EDTA, pH 7.5 (a–d), 0.15 M NaCl, pH 7.5 (e–i), and 0.5 M NaCl, pH 7.5 (j–o). (a, e, and j) $H2A_{n,n}$, control native H2A.1 from this chromatin in each respective ionic strength condition which had no trypsin added; (b, c, and d) 2.25, 7, and 21 h of trypsin treatment, respectively; (f, g, h, and i) 0.25, 0.75, 2.25, and 7 h of trypsin treatment, respectively; (k, l, m, n, and o) 0.25, 0.75, 2.25, 7, and 21 h of trypsin treatment, respectively.

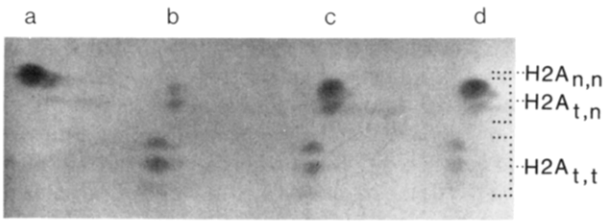


FIGURE 7: H2A:H2B dimer–DNA complexes were trypsin treated, and the H2A.1 cleavage products were analyzed. (a) $H2A_{n,n}$, control native H2A.1 from the dimer–DNA complex which was not trypsin treated. (b, c, and d) H2A.1 peptides produced when the H2A:H2B dimer–DNA complex was treated with trypsin for 100, 25, and 5 min, respectively.

of $H2A_{t,n}$ from $H2A_{n,n}$ in the early stages of digestion. Later in the time course, these forms were converted to a single form of $H2A_{t,t}$ (data not shown).

Discussion

In this report, we have utilized a combination of gel electrophoretic systems to demonstrate that distinct patterns of H2A cleavage by trypsin are affected upon the association of this histone into the various levels of chromosome organization, i.e., H2A:H2B dimer, core histone octamer, chromatin, and nuclei. Several species of trypsin-limit peptides, each of which is quite homogeneous in mobility on AUT–AUC and Na-$DodSO_4$ gel systems, are identified. Although amino acid sequencing or fingerprint analysis of the tryptic peptides would be necessary to precisely identify the sites of cleavage, we were able through careful analysis of the electrophoretic mobilities of the peptides produced to estimate the probable sites of trypsin cleavage. More importantly, the precursor–product relationships of the peptides produced by trypsinization of each of the substrates were analyzed. Use of the H2A-specific protease, which cleaves between $valine_{114}$ and $leucine_{115}$, allowed us to specifically monitor the presence or absence of this site in the trypsin-limit peptides produced by the trypsinization of the core histone octamer. In this way, we were able to make a number of conclusions about the accessibility to trypsin of the amino- and carboxy-terminal domains of H2A in the various substrates tested.

The sequence of the 129 amino acids of calf thymus H2A has been completely determined (Skandrani et al., 1972; Sautiere et al., 1974). As observed by Bradbury and co-workers (Bradbury et al., 1975), there is nonuniform distribution of residues in H2A. The amino-terminal region (residues 1–32) of this protein has a net positive charge of 10+; it has 10 helix-destabilizing residues and only 3 apolar residues. The stretch from residues 33 to 116 is apolar in nature and is only slightly positive in charge (4+ for 83 residues). The carboxy-terminal 13 amino acids (residues 117–129) are very basic with a net positive charge of 6+, and 3 of the 13 residues are helix destabilizing. Extensive proton magnetic resonance spectroscopic and circular dichroism studies indicate that the structured regions of H2A in the H2A:H2B dimer extend from residue 33 to residue 95 and possibly as far as residue 116. The amino- and carboxy-terminal ends of H2A are unstructured and project from the central globular region of the H2A:H2B dimer (Moss et al., 1976). There are seven possible trypsin cleavage sites on the amino-terminal side of H2A with the innermost site being between residues 20 and 21, while on the carboxy-terminal side, there are four possible cleavage sites with the innermost between residues 118–119. Bohm et al. (1980) claim that the principal cleavage points are at the Arg_{11}–Ala_{12} bond and at the Lys_{118}–Lys_{119} bond.

The difference in mobilities between $H2A_{t,n}$ and $H2A_{t,p}$ is equivalent to that between $H2A_{n,n}$ and $H2A_{n,p}$ (Figures 2–4). Therefore, native H2A trypsinized while organized in the octamer not only retains the site for the H2A-specific protease but also has not been significantly cleaved on its carboxy-terminal side. This region of the molecule seems to be protected from the action of trypsin when the association of the dimer with the tetramer is favored. T. H. Eickbush and E. N. Moudrianakis (unpublished experiments) have found that the H2A-specific protease site is similarly protected from cleavage by this enzyme in conditions which favor the octamer form. They also found that removal of the 15 C-terminal amino acids from purified H2A does not affect the interaction of H2A with H2B in forming a dimer, but this type of modified dimer has a significantly reduced association with the $(H3:H4)_2$ tetramer. Native H2A:H2B dimers were able to effectively compete out H2A-specific protease-cleaved dimers for association with sites on $(H3:H4)_2$ tetramers and were consequently eluted in the core complex region of the chromatographic profile of a Sephadex G100 column run in 2 M NaCl, pH 7.5, 4 °C. The H2A-specific protease-cleaved dimers were preferentially eluted at the position of free dimer. In this study, the trypsin-treated form of octamer was found to elute from a Sephadex G100 column at a later point than does native octamer (data not shown). The position of elution for this single peak of "trypsinized" octamer is that expected for a histone octamer which has lost the trypsin-susceptible regions of its component polypeptides. These regions make a contribution not only to the mass of the complex but also, most significantly, to its Stokes' radius (Eickbush & Moudrianakis, 1978). $NaDodSO_4$–polyacrylamide gel electrophoresis shows that the peak of trypsinized octamer contains all of the major trypsin-resistant peptides of each of the core histones (data not shown). The trypsin forms of the dimer and the tetramer have therefore retained their ability to associate in these conditions. This provides another piece of evidence that the carboxy-terminal region of H2A is protected from cleavage by trypsin.

The next phase of this study involved assessing the order of cleavage of the amino- and carboxy-terminal ends of H2A in nuclei and chromatin. It is well established that extensively trypsinized chromatin and core particles contain a distinct, reproducible set of limit peptides for each of the core histones and retain most of the characteristics of their native counterparts (Weintraub & Van Lente, 1974; Lilley & Tatchell, 1977; Whitlock & Stein, 1978). This might be explained in several ways. A number of studies have indicated that the histone tails may not be as tightly bound to DNA in chromatin and within the core particle as once thought. A proton magnetic resonance study by Cary et al. (1978) gave evidence that the amino- and carboxy-terminal tails of H2A and H2B in the core particle were free in 0–0.2 M NaCl. Palter & Alberts (1979) found that if native or trypsinized core histones were reconstituted onto high molecular weight DNA–cellulose and then eluted by a salt gradient, the concentration of salt necessary for elution was the same in each case. Interestingly, Mathis et al. (1978) found that even extensive histone acetylation did little to affect the ability of the core histone octamer to slide along the DNA or to exchange between DNA molecules. Trypsin susceptibility, therefore, may not simply be the result of the exposure of the lysine- and/or arginine-rich terminal regions while bound to the DNA but rather an indicator of their freedom to interact outside of the immediate domain of the nucleosome.

Bohm and co-workers (Bohm et al., 1980–1982) have fractionated and characterized the major trypsin-limit peptides of each of the core histones from trypsinized chicken eryth-

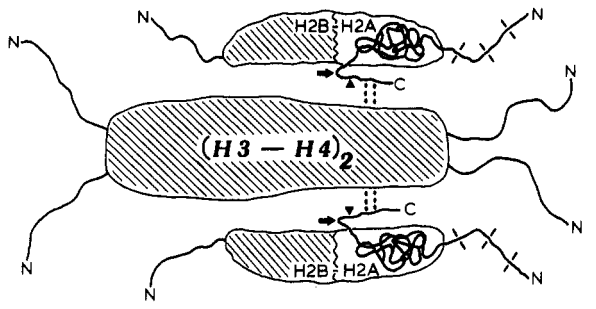


FIGURE 8: Diagrammatic representation of the amino and carboxy termini of the core histones within the histone octamer. The three probable sites of trypsin action on the amino terminus of H2A are indicated by the three hatched areas. These sites are available for cleavage in all forms of dimer, i.e., when free in solution, when assembled into the histone octamer, and while bound to DNA. One the carboxy-terminal end of the H2A, there exists at least one site cleavable by trypsin (▲) and a specific site cleavable by our H2A-specific protease (→). Neither of these sites is cleaved, however, when the dimer is tightly assembled in the octamer form by a limited number of hydrogen bonds (dotted lines). Upon disruption of these bonds and opening of the dimer–tetramer contact interface, both of these sites are available for proteolysis.

rocyte nuclei. The final trypsin-limit digest peptide of H2A was found to be residues 12–118 of the original 128 amino acid length. The final tryptic peptide of H2A that we have observed in this study was found to be produced by the sequential cleavage of the amino-terminal end of the molecule ($H2A_{t,n}$) and then finally the carboxy-terminal end ($H2A_{t,t}$; Figures 2–6). Careful analysis of the electrophoretic mobilities of the peptides produced indicates that the final cleavage sites observed here are most probably those identified by Bohm et al. (1980). There are several possible explanations for the ordered cleavage of H2A by trypsin as observed in this study. The carboxy-terminal region of the molecule may be less amenable to cleavage by trypsin than the amino-terminal region because either the carboxy-terminal region of H2A is protected in the octamer, presumably because of the dimer–tetramer interaction, or its secondary structure is modified in such a way that it is less efficiently cleaved. The latter possibility may explain the sequential cleavage of H2A in the H2A:H2B dimer free in solution. In the case of chromatin, there is an indirect piece of evidence that the carboxy-terminal region of H2A might normally be accessible to enzymatic modification(s). Wu et al. (1981) have observed that the ubiquitin moiety of protein A24, which is attached at residue 119 of histone 2A (Goldknopf & Busch, 1977), is in rapid equilibrium with the pool of free ubiquitin in both dividing and nondividing cells. Therefore, either of the reasons given above may explain the ordered cleavage of the terminal regions of H2A in chromatin. Finally, the initial removal of the amino-terminal side from this particular histone or from each of the core histones in concert may somehow loosen the contact of the carboxy terminus of H2A with either the dimer and/or the tetramer in the octameric structure when bound to DNA in chromatin.

When chromatin is trypsinized, three forms of $H2A_{t,n}$ differing slightly in electrophoretic mobility are observed at early times of digestion (Figure 6), and these are progressively converted to the fastest mobility form of $H2A_{t,n}$ and then to $H2A_{t,t}$. The heterogeneity of $H2A_{t,n}$ and $H2A_{t,t}$ observed in this case was also seen when the purified H2A:H2B dimer alone was reconstituted to DNA prior to trypsin treatment (Figure 7). The observation of single peptides representing $H2A_{t,n}$ and $H2A_{t,t}$ when free H2A:H2B dimer in solution was trypsin treated indicates that the binding of the histones to DNA influences the way in which trypsin cleaves the susceptible regions of H2A. This pattern of sequential cleavage provides further support for the suggestion of Bohm et al. (1982) that all or most trypsin cleavage sites outside of the innermost amino-terminal trypsin site are susceptible to cleavage and that it is not the result of the exposure of a bridgehead between two protected domains. This is also quite likely to be the case for the trypsin cleavage of the carboxy-terminal region as well.

In conclusion, the combined use of trypsin and the H2A-specific protease and the resolution of the H2A cleavage products on a series of electrophoretic systems have allowed us to ascertain that although the amino-terminal domain of H2A is very trypsin sensitive in all conditions tested, the carboxy-terminal domain of H2A is protected from the action of trypsin when organized in the octamer in solutions of high ionic strength. Dissociation of the octamer exposes this region of H2A to trypsin, and the pattern of H2A cleavage observed is similar to that observed when free dimer is treated with trypsin (Figure 8). Similarly, the carboxy-terminal domain of H2A organized in chromatin is less readily cleaved by trypsin than the amino-terminal side of the molecule. These observations indicate that the interaction of the dimer with the tetramer and DNA in chromatin is not as tight as when the dimer has associated with the tetramer to form the octamer in solutions of high ionic strength. Work is in progress to probe the regulation of the association of the octamer via modification of the contact interface between dimer and tetramer and to assess the possible role of this interface in the regulation of chromatin function.

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Dynamic Structure and Phase Behavior of Dimyristoylphosphatidylethanolamine Bilayers Studied by Deuterium Nuclear Magnetic Resonance†

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ABSTRACT: The dynamic structure of dimyristoylphosphatidylethanolamine bilayers has been studied by deuterium nuclear magnetic resonance spectroscopy of the perdeuterated *sn*-2 chain. The order parameter profile of the lipid chains in the fluid phase is qualitatively similar to that found for other phospholipids, but the order parameter plateau is ca. 15% higher than found for dimyristoylphosphatidylcholine at a comparable reduced temperature. The chains of dimyristoylphosphatidylethanolamine undergo a segmental motion in the gel phase, which for segments close to the end of the chain approximates continuous axial diffusion. In the phase-transition region, spectra are observed that can be best described in terms of the interconversion of coexisting lipid phases through the transition.

The lipid composition of biological membranes shows diversity both in the hydrocarbon-chain content and in the different phospholipid head-group classes [see, e.g., Marsh (1975)]. The hydrocarbon-chain composition is normally regulated such that the lipids are in a fluid, liquid-crystalline state at physiological temperatures (Chapman, 1975). The functional implications of the lipid head-group diversity are less clear. One effect is presumably to modulate the surface properties of the membrane, especially in the case of negatively charged lipid head groups. Another possibility is that the degree of fluidity and chain ordering may vary between the different lipid classes, and this could provide a mechanism whereby the dynamic properties of a fluid membrane could be finely adjusted by varying the phospholipid head-group composition. If this mechanism were operative, spatially differentiated areas of varying fluidity might also be created by fluid–fluid phase separation between different lipid classes. This would provide a method of regulating membrane dynamics in a progressive manner, without the disruptive effects associated with the gel–fluid bilayer phase transition or the pronounced ordering effects produced by cholesterol.

Two of the principal lipid classes in mammalian membranes are the zwitterionic lipids phosphatidylcholine and phosphatidylethanolamine. In addition, phosphatidylethanolamine is the major membrane phospholipid in many microorganisms. In the present paper, we have compared the chain ordering in phosphatidylethanolamines with that in the much more studied phosphatidylcholines, using deuterium nuclear magnetic resonance (NMR).[1] It is found, by comparing the dimyristoyl derivatives with a perdeuterated *sn*-2 chain, that the chain ordering in phosphatidylethanolamine is significantly greater than in phosphatidylcholine at the same reduced temperatures. In addition, we have investigated the dynamic properties of the dimyristoylphosphatidylethanolamine chains both in the gel phase and through the gel-to-fluid phase transition.

Materials and Methods

Perdeuterated myristic acid was obtained from Merck Sharp & Dohme, Canada Ltd. 1-Myristoyllysophosphatidylcholine (Calbiochem, Giessen, West Germany) was acylated with the carboxylimidazole complex of perdeuterated myristic acid

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[1] Abbreviations: DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance.

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