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On the Location of Histones H1 and H5 in the Chromatin Fiber

Studies with Immobilized Trypsin and Chymotrypsin

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The location of linker histones H1 and H5 in chicken erythrocyte chromatin was studied as a function of the fiber structure by the use of proteolytic enzymes immobilized onto Immobilon membranes. The immobilization of trypsin and chymotrypsin creates proteolytic probes, specific respectively to the terminal portions of the molecules or to the phenylalanine in the globular domain, that are incapable of penetrating into the interior of the condensed fiber. The chromatin fiber was studied in three different conformations: open zig-zag (in Tris buffer), closed zig-zag (upon addition of 10 mM-NaCl), or 30 nm fiber (upon addition of 0·35 mM-$MgCl_2$). The results from digestion experiments performed on linker histones either in chicken erythrocyte chromatin, or free in solution or bound in mononucleosomes revealed several features relevant to linker histone location: (1) histone H5 is more protected than histone H1 in the fiber; (2) the N and C-terminal portions of histone H1 do not change their accessibility, and hence their location, upon compaction of the fiber; this behavior of H1 is in contrast to that of histone H5, whose tails become significantly internalized in the 30 nm fiber; (3) phenylalanine in the globular domain of both H1 and H5 is inaccessible (buried) both in the fiber and in the mononucleosomal particle. Sedimentation velocity measurements performed during the course of trypsin digestion demonstrate that the conformation of the fiber is highly sensitive to even a few cuts in some of the linker histone molecules; hence, the linker histones are an important factor in the organization of the fiber in all its different condensation states.

Keywords: chromatin; linker histones; higher order structure; immobilized trypsin and chymotrypsin; analytical centrifugation

1. Introduction

It is now widely accepted that most chromatin in the eukaryotic nucleus is organized into 30 nm fibers (van Holde, 1988). A variety of models have been proposed for the 30 nm fiber (for reviews, see Felsenfeld & McGhee, 1986; Sayers, 1988; Thoma, 1988; Freeman & Garrard, 1992), based mainly on results from physical studies and electron microscopic observations; however, none of them is universally accepted. A serious obstacle in resolving the higher-order structure of the chromatin fiber lies in our lack of knowledge as to where and how the linker histones (the variants of the lysine-rich histone H1 and H5) are located.

The linker histones have long been implicated in the formation and/or maintenance of the higher-order structure (e.g. see Thoma *et al.*, 1979; Thoma & Koller, 1981; Allan *et al.*, 1981, 1986; Thoma, 1988). The linker histones are represented in each cell by a family of closely related molecular species (Cole, 1987). The H1 complement can vary during development and differentiation, with some specific H1 subtypes present only in certain cell types. The best studied example of a cell-specific H1 subtype is histone H5, present in nucleated erythrocytes of birds and some fish (Neelin *et al.*, 1964; Miki & Neelin, 1975). This histone has been implicated in the process of terminal differentiation as a factor in the shutting down of transcription and replication in the mature erythrocyte.

The linker histones are characterized by a highly asymmetrical distribution of the various types of amino acid residues along the polypeptide chain. They adopt, when dissolved in the presence of salt, a well-defined, three-dimensional structure that

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consists of a short, randomly coiled basic N-terminal tail, an apolar globular domain of about 80 amino acid residues and a long, basic, randomly coiled C-terminal tail (Hartman *et al.*, 1977; Aviles *et al.*, 1978). The different structural domains are thought to perform different roles in structuring the nucleosome and the fiber. Thus, while the globular domain seals off the two turns of DNA in the nucleosome (Allan *et al.*, 1980), it is the C-terminal tail that interacts with DNA to form higher-order structures (Allan *et al.*, 1986; for a review, see Zlatanova & Yaneva, 1991). The function of the short N-tail is less well known; it is believed that it serves to place precisely, or anchor, the globular domain with respect to the nucleosome (Allan *et al.*, 1986).

The issue of the location of the linker histones in the fiber is of immediate importance in understanding the higher-order structure of chromatin in the nucleus. Do they lie within the fiber, on its surface, or both? The data, obtained thus far mainly from immunochemical studies (for a review, see Zlatanova, 1990), are highly controversial. In an attempt to obtain some insight into the location of the linker histones in chromatin fibers of different conformations and also to see whether differences exist between locations of the two major, presumably functionally different, linker histone subtypes in chicken erythrocyte chromatin, we used immobilized proteolytic enzymes as probes for the accessibility of the histone molecules. Immobilization serves to preclude proteolytic attack at sites located inside an ordered condensed structure and thus permits discrimination between external and internal localization. The proteolytic enzymes were chosen so as to distinguish between the location of the terminal portions of the molecules (trypsin) and the globular domains (chymotrypsin).

2. Materials and Methods

(a) *Enzyme immobilization on membranes*

Diphenyl carbamyl chloride-treated trypsin (Sigma type XI) and α-chymotrypsin (Sigma type II) were immobilized on Immobilon (Millipore) membranes. Immobilization was carried out at 4°C; a solution containing 1 mg of enzyme/ml of coupling buffer (0·5 M-potassium phosphate, pH 7·4) was incubated with 24 mm diameter disks of the membrane for 1 h. Membranes were incubated in capping solution (0·1% (w/v) gelatin in 1·0 M-sodium bicarbonate, pH 9·5) for 2 h and finally incubated for 30 min in wash solution (0·01 M-sodium phosphate (pH 7·4) containing 0·1% (v/v) Tween-20). In all incubations, solutions were agitated to keep the membranes away from the walls of the vessels. Membranes were then dried on Whatman filter paper and stored in sealed Petri dishes at 4°C.

(b) *Preparation of chromatin*

Chromatin was prepared as described by Ausio *et al.* (1989). All buffers were made 0·1 mM in PMSF† immediately before use. Fresh chicken blood was centrifuged at low speed at 4°C. The cell pellet was extensively washed in membrane lysis buffer (0·1 M-KCl, 50 mM-Tris·HCl (pH 7·5), 1 mM-$MgCl_2$, 0·5% (v/v) Triton X-100) until the pellet was white. The pellet was resuspended in micrococcal nuclease digestion buffer (0·1 M-KCl, 50 mM-Tris·HCl (pH 7·5), 1 mM-$CaCl_2$) and mildly digested with micrococcal nuclease (Worthington). The nuclear suspension was then hypotonically lysed in 0·25 mM-EDTA (pH 7·5) and the soluble chromatin was extensively dialyzed *versus* 10 mM-Tris·HCl (pH 7·5) and stored at −80°C. Soluble chromatin was analyzed for histone content in 15% (w/v) polyacrylamide/SDS slab gels in the discontinuous buffer system described by Laemmli (1970). The length of the DNA in the chromatin was checked by electrophoresis on 1% (w/v) agarose in TAE buffer (40 mM-Tris·HCl (pH 8·0), 40 mM-acetic acid, 1 mM-EDTA; Maniatis *et al.*, 1982). Finally, the sedimentation behavior of the chromatin was determined by analytical ultracentrifugation (see below).

(c) *Preparation of histones H1 and H5*

H1 and H5 were prepared as described by Garcia-Ramirez *et al.* (1990). The soluble chromatin was made 0·35 M with respect to NaCl, and linker histones were stripped from chromatin by addition of CM-Sephadex C-25. The resin was then removed by low-speed centrifugation. The resin cake was washed in 0·35 M-NaCl, 10 mM-Tris·HCl (pH 8·8) and loaded onto a CM-Sephadex C-25 column previously equilibrated in the same buffer. The linker histones were eluted by a linear salt gradient of 0·35 M to 1·6 M-NaCl, 10 mM-Tris·HCl (pH 8·8). Protein content was analyzed on SDS/polyacrylamide gels. Finally, the histones were extensively dialyzed *versus* 10 mM-Tris·HCl (pH 7·5) and stored on ice.

(d) *Preparation of nucleosomes*

Nucleosomes containing the full complement of histones were prepared with minor modifications of the method of Ausio *et al.* (1989). Soluble chromatin at 7·5 mg/ml was made 1·0 mM with respect to $CaCl_2$ and digested with 2 units of micrococcal nuclease/ml (Worthington) for 5 h at 25°C. Digestion was checked by electrophoresis on 1·5% agarose in TAE buffer (Maniatis *et al.*, 1982). Enzyme digestion was stopped by adding EDTA to 10 mM, cooling to 4°C, and dialyzing *versus* 10 mM-Tris·HCl (pH 7·5), 10 mM-EDTA, 0·1 mM-PMSF. The digested sample was loaded onto a Sephacryl S-300 column (200 cm × 5 cm) that had been equilibrated with the same buffer. Fractions were checked for free DNA and nucleosome monomer content on 4% native polyacrylamide gels as described by Ausio *et al.* (1989). DNA size was monitored by incubating the sample in an equal volume of dissociation sample buffer (0·4% (w/v) SDS, 0·04% (w/v) bromophenol blue, 20% (v/v) glycerol, 40 mM-Tris·HCl (pH 8·0), 1 mM-EDTA; Juan Ausio, personal communication) for 30 min at 37°C before loading onto 4% native polyacrylamide gels. Histone content was checked on SDS/polyacrylamide gels. Column fractions containing nucleosome monomers with a DNA distribution of 200(±10) bp and both core and linker histones were extensively dialyzed *versus* 10 mM-Tris·HCl (pH 7·5).

(e) *Enzyme digestions*

Chromatin samples were removed from the −80°C freezer and thawed. Precipitation material was removed

† Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; bp, base-pair(s).

by centrifugation at 10,000 *g* in a microcentrifuge (Eppendorf) for 5 min. Digestion was performed at a relatively low concentration (0·1 mg DNA/ml) to prevent aggregation artifacts. Digestions of nucleosomes were also at 0·1 mg/ml. Digestions of linker histones free in solution were at 0·1 mg protein/ml. Enzyme-immobilized membranes were immersed in the reaction solution 15 min prior to the addition of substrate. Samples were digested in 10 mM-Tris·HCl (pH 7·5) or in the same buffer containing either 10 mM-NaCl or 0·35 mM-$MgCl_2$. Samples (0·5 ml) were taken at the indicated times, frozen, lyophilized with a Speedvac (Savant), resuspended in 2×SDS sample buffer and analyzed on SDS/polyacrylamide gels.

(f) *Sedimentation velocity*

Sedimentation velocity measurements were done as described (Ausio *et al.*, 1989) on a model E analytical ultracentrifuge (Beckman) equipped with a photoelectric scanner and a multiplexer. Soluble chromatin had an A_{265} of 0·7 to 0·9 and was centrifuged at 12,000 revs/min at 20(±1)°C. The temperature was kept constant within each run to within 0·1 deg.C using the RTIC temperature control unit. Data were collected on a computer interfaced with digitized scanner output (Demeler, 1992) and analyzed by the method of van Holde & Weischet (1978) to determine the integral distribution of the sedimentation coefficients.

(g) *Quantitative analysis of histone gels*

SDS/polyacrylamide gels or their photographic negatives were scanned on a soft laser scanning densitometer (Zeineh). Densitograms were enlarged on a photocopier and the peaks were cut out and weighed. To compensate for different loadings, the amount of linker histones was normalized to the amount of histone H4 in each lane. Chromatin-contained histone H4 was chosen as a reference protein as it is relatively stable to enzymatic cleavage and under the mild digestion conditions used in this study (see Results) its amount remained constant with time of digestion. Results are presented as percentage of the amount (relative to H4) of the respective linker histone at the zero time-point of digestion. In the case of linker histones free in solution, the amount of H1 and H5 at the zero time-point was taken as 100%. To increase the accuracy of determination of the (relative) amounts of linker histones at the zero time-point, several zero time-points were loaded on each gel and the determinations averaged. The peaks were approximated as Gaussian curves as suggested by Staynov & Crane-Robinson (1988). We estimate an accuracy of estimation of the area under the peaks better than ±20%. Because measurements of this kind are subject to significant experimental error, each experiment was repeated a number of times.

3. Results

(a) *Experimental approach*

The experiments reported here were designed to address the question of whether and to what extent the location of the linker histones in chicken erythrocyte chromatin changes as a function of the transition of the chromatin fiber from extended to progressively more condensed conformations. Immobilized proteolytic enzymes, trypsin and chymotrypsin, were used as probes to compare the accessibility of the linker histones in the fiber, free in solution or in isolated mononucleosomal particles. The use of immobilized endopeptidases possesses several advantages over the use of soluble enzymes. (1) The reaction can be stopped instantly by simply removing the membrane from the protein-containing solution. (2) It allows extremely mild digestion conditions so that the digestion of H1 and H5 can be monitored before any significant digestion of the core histones takes place; parallel physical studies can yield information concerning the structural changes in the fiber accompanying the initial digestion of a fraction of linker histone molecules. (3) The immobilization on a solid surface prevents the enzyme molecule from penetrating into the interior of the fiber, even at times when the fiber is "breathing", i.e. temporarily and locally opening and closing. This, in turn, avoids the necessity to fix fiber structure against breathing, using chemical cross-linking agents such as glutaraldehyde. Chemical fixation of the fiber is highly undesirable as it is poorly controllable, and it may lead to artifacts of its own, as shown in immunochemical studies (for a review, see Zlatanova, 1990; see also discussion in Banchev *et al.*, 1990). Control experiments were performed to make sure that the enzymes remained attached to the membrane even after prolonged incubation under the conditions used for digestion.

All experiments were performed on large molecular mass soluble chromatin fragments, containing about 75±30 nucleosomes (the average S value in Tris determined by analytical centrifugation was about 58±7; the average molecular mass of the DNA estimated by agarose gel electrophoresis was $15(\pm 6)\times 10^3$ bp). No material of DNA size below 2×10^3 bp was present in the preparations. The use of large molecular mass fragments was required in order to: (1) avoid possible end-effects that might significantly distort data obtained on oligonucleosomes; and (2) ensure proper formation of condensed structures (at least 6 nucleosomes are necessary to form higher-order structure (Bates *et al.*, 1981; McGhee *et al.*, 1983). Control experiments showed that our chromatin preparations did not contain significant endogenous proteolytic activities.

To study the accessibility of chromatin-contained linker histones in fibers of different structural characteristics, we compared the proteolytic digestion patterns of chromatin dissolved in 10 mM-Tris without salt, with addition of 10 mM-NaCl, or with additions of 0·35 mM-$MgCl_2$. As well documented in the literature (for a review, see Thoma, 1988), in the absence of salts, the chromatin fiber exists as an extended open zig-zag, with the linker DNA entering and exiting the nucleosomes on the same site; addition of salt to 5 to 10 mM-NaCl leads to closing of the zig-zag, bringing the nucleosomes in close proximity. Finally, in the presence of 0·35 mM-$MgCl_2$ the fiber condenses into the 30 nm fiber structure (Ausio *et al.*, 1984), indistinguishable from that observed in the nucleus under physio-

logical salt conditions. The choice of Mg^{2+} instead of the more commonly used Na^+ for chromatin condensation was made in order to avoid the redistribution of linker histones known to occur at the NaCl concentrations necessary to achieve chromatin condensation (Caron & Thomas, 1981) and was based on a careful physical study of the condensation process driven by either Na^+ or Mg^{2+} (Ausio *et al.*, 1984). Sedimentation velocity measurements showed that the median sedimentation coefficient of the chromatin fiber was about 57, 85 and 140 S in Tris, 10 mM-NaCl and 0·35 mM-$MgCl_2$, respectively (Fig. 10), as expected from gradual condensation of the fiber.

Addition of divalent ions of concentrated chromatin solutions often brings about aggregation, and it was important to work under conditions where no significant precipitation of chromatin took place during the course of the experiment. This was achieved by using relatively diluted chromatin solutions (about 100 μg of DNA/ml) and keeping the Mg^{2+} concentration at the lowest value that would still assure condensation. Measurements of the A_{260} of the chromatin preparations at different times during proteolytic digestion following removal of any precipitated material by centrifugation showed that even eight hours after the addition of Mg^{2+} only about 50% of the material had precipitated.

(b) *Trypsin digestion*

(i) *Trypsin digestion of long chromatin*

Trypsin, either free in solution or immobilized on collagen membranes, has been widely used in studies that attempted to correlate chromatin fiber unfolding with digestion of H1 and core histones (for reviews, see Böhm & Crane-Robinson, 1984; Hacques *et al.*, 1990). Because the N and C-tails of the molecules of the linker histones are rich in lysine and arginine residues, trypsin degrades them quickly, giving rise in each case to a relatively stable fragment encompassing the central structured globular domain of the histone molecule (Allan *et al.*, 1980).

A typical pattern of immobilized trypsin digestion of long chromatin fibers in the three structural states is presented in Figure 1. It is clear that the digestion was extremely mild: even at eight hours only about 50% of the linker histone molecules had been digested. At the same time the trypsin-resistant globular domains of both H1 and H5 became evident, as bands migrating below H4. Because we are confining our studies to early digestion time-points, the relative kinetics of digestion of H1 and the core histones were difficult to follow; in accordance with most published data (Böhm & Crane-Robinson, 1984), we found that H3 was the histone attacked immediately following degradation of some of the linker histone molecules, as judged by the appearance of the proteolytic fragment P1′, situated just above H4. As attempts to localize linker histones with respect to the fiber higher-order structure will be compromised in preparations

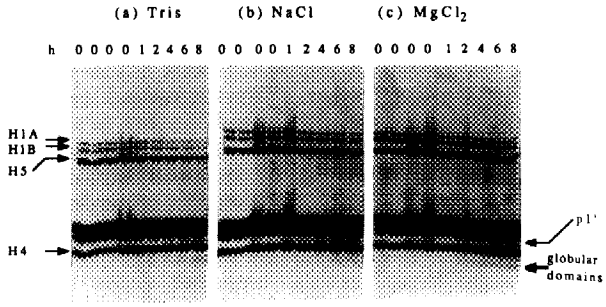


Figure 1. SDS/polyacrylamide gel electrophoretic patterns of long chromatin digested with immobilized trypsin at 25 °C. Chromatin was dissolved in (a) 10 mM-Tris·HCl (pH 7·5) or in the same buffer containing (b) 10 mM-NaCl or (c) 0·35 mM-$MgCl_2$. Time of trypsin treatment (h) is denoted above the lanes. The positions of the 2 major H1 fractions resolvable by electrophoresis are marked by arrows to the left, as are the positions of histones H5 and H4. The positions of the globular domains of H1 and H5, as well as that of the major digestion product of histone H3 (P1′) are marked to the right.

where the fiber is destroyed due to proteolysis of the core histones, specific care was taken to include in the analysis of the linker histone accessibility only time-points of digestion before visible degradation of the core histones had occurred.

The digestion patterns were quantified by scanning of the gels and determining the area under the histone peaks. To compensate for possible different protein loadings in the individual lanes, all values for H1 and H5 content were normalized to the amount of H4 present in the respective lanes (H4 was used for normalization as there was no sign of degradation of this histone even late during digestion and as it was well separated from the other histones in electrophoretic gels). The results of such quantification are presented in Figure 2. As can be seen, histone H5 was somewhat more protected than histone H1 under all three conditions tested: Tris, 10 mM-NaCl and 0·35 mM-$MgCl_2$. In the more extended conformations of the fiber (Tris, 10 mM-NaCl), there was only a slight albeit highly reproducible, protection; on the other hand, H5 was much more protected in 0·35 mM-$MgCl_2$. That the higher protection of H5 in the condensed fiber was due to the formation of the higher-order structure is especially evident from comparisons among the three conditions for each histone: while H1 seemed to be equally accessible to digestion under all conditions, H5 apparently became more protected only in the Mg^{2+}-containing solution. The data shown in Figures 1 and 2 are representative of those observed in six independent experiments using several different preparations of immobilized trypsin.

(ii) *Trypsin digestion of free linker histones in solution*

There are possible trivial explanations of the results reported above. First, it could be that H5 is

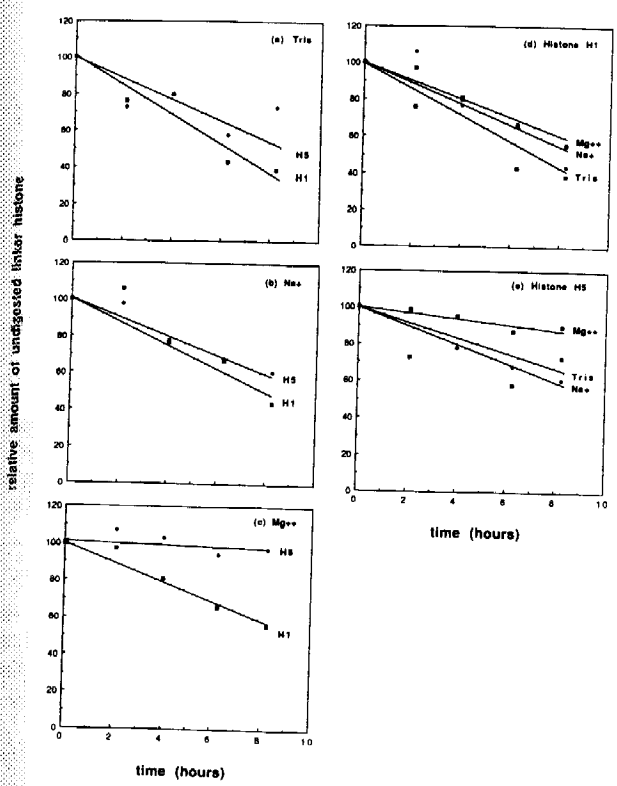


Figure 2. Quantification of the trypsin digestion patterns of long chromatin presented in Fig. 1. The electrophoretic gels were scanned and quantification was performed as described in Materials and Methods. For easier interpretation the data are presented as pairwise comparisons between histones H1 and H5 under the 3 conditions: (a) 10 mM-Tris·HCl (pH 7·5), (b) 10 mM-NaCl and (c) 0·35 mM-$MgCl_2$. Additionally, the curves for each linker histone under the 3 conditions are plotted on the same graph: (d) histone H1 and (e) histone H5. The values on the ordinate represent the amount of undigested linker histone relative to the amount of histone H4, the corresponding values for the zero time-point being taken as 100%.

intrinsically more resistant than H1 to trypsin digestion even when free in solution. Second, it is possible that the presence of magnesium ions specifically decreases the rate of digestion of H5 in comparison with that in Tris or in NaCl. To check for these possibilities, an equimolar mixture of purified H1 and H5 was subjected to digestion with immobilized trypsin under the salt conditions used to differently structure the long chromatin fiber. The results for Tris and $MgCl_2$ are presented in Figure 3. Histones H1 and H5 were digested at indistinguishable rates either in the absence of salt or in the presence of 0·35 mM-$MgCl_2$. Similar digestion curves were obtained using 10 mM-NaCl (not shown). Thus, the differences observed with long chromatin-contained linker histones reflect features of accessibility to the enzymatic probes related to chromatin structure rather than differences inherent in the interaction of the free histone molecules themselves with the enzyme.

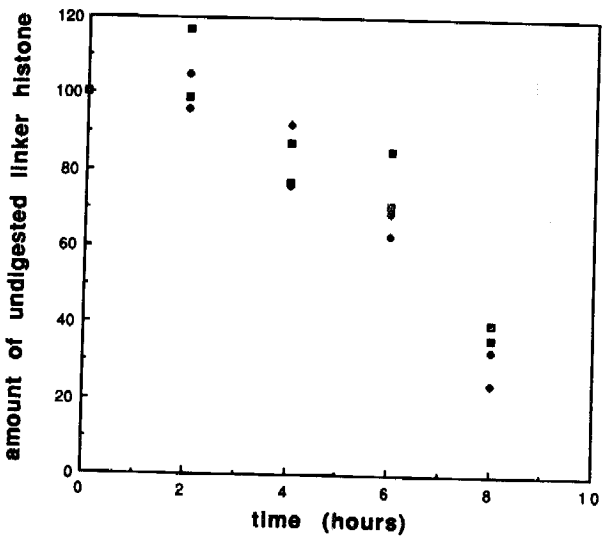


Figure 3. Quantification of the digestion of the linker histones free in solution in Tris·HCl (pH 7·5) and in 0·35 mM-$MgCl_2$ at 25°C. An equimolar mixture of purified H1 and H5 was subjected to trypsinization, run on SDS/polyacrylamide gels and the amount of linker histones remaining at the position of the intact molecules was quantified by setting the amount present at the start of digestion as 100%. The symbols are: (□), H1 in Tris; (◆), H1 in Mg^{2+}; (■), H5 in Tris; (◇), H5 in Mg^{2+}.

(iii) *The linker histones are structured in all conformations of the fiber*

Any study aimed at determining the role or location of the linker histones in the chromatin fiber should be performed under conditions under which the histone is structured in its native tertiary conformation. It is known that these molecules exist as random coils in solution in the absence of salt and that the transition of freely dissolved linker histones to their organized structures requires the addition of salt (100 mM-NaCl; Smerdon & Isenberg, 1976). Consistent with this, our control digestion experiments with the free linker histones did not show any evidence for a trypsin-resistant protein core under any of the three ionic conditions studied. Therefore, it was important to make sure that the linker histones were structured while chromatin-bound. That binding of H1/H5 to DNA in chromatin might mimic higher salt concentrations has long been suspected, since some of the positive charges on the molecules (particularly those in the C and N-terminal tails) are neutralized by interaction with the DNA (see the discussion by Thoma, 1988). In fact, experiments with H1/DNA model systems have shown that a trypsin-resistant folded globular domain could be detected at salt concentrations as low as 15 mM-NaCl (Clark & Thomas, 1986).

A careful examination of the trypsin digestion patterns (Fig. 1) revealed the gradual appearance of two closely migrating bands below H4, corresponding to the globular domains of H1 and H5. The rate of accumulation of these domains was proportional to the rate of disappearance of the

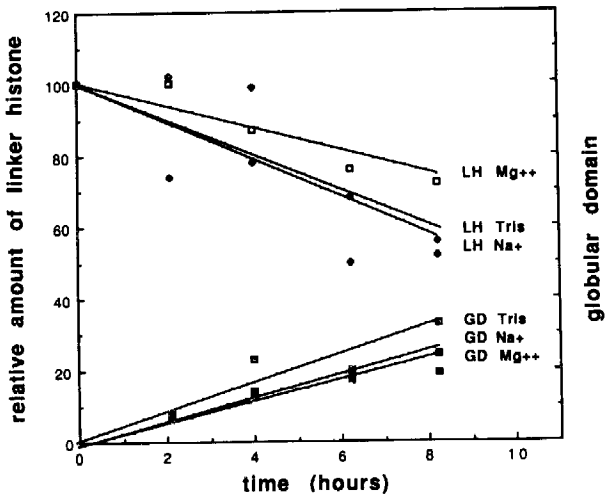


Figure 4. Time-course of digestion of the chromatin-contained linker histones (LH) and appearance of the globular domain (GD) under the 3 different salt conditions: 10 mM-Tris·HCl (pH 7·5), 10 mM-NaCl and 0·35 mM-$MgCl_2$ (marked as Tris, Na^+ and Mg^{++} on the graph). The data for the digestion of histones H1 and H5 have been presented as common curves; the same holds true for the globular domains of the 2 proteins. The figures for the globular domains represent the proportion of total intact linker histones that appears as globular domains (it was considered that the globular domain encompasses around 40% of the size of the molecule of an "averaged" linker histone molecule).

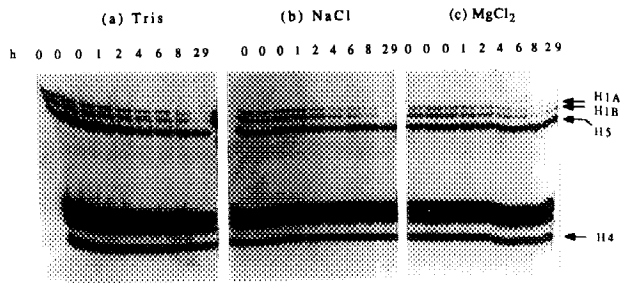


Figure 5. SDS/polyacrylamide gel electrophoretic patterns of long chromatin digested with immobilized chymotrypsin. Chromatin was dissolved in (a) 10 mM-Tris·HCl (pH 7·5) or in the same buffer containing (b) 10 mM-NaCl or (c) 0·35 mM-$MgCl_2$ and the digestion was carried out at 37 °C. Time of chymotrypsin treatment (h) is denoted above the lanes. The positions of H1 (see the legend to Fig. 1), H5 and H4 are marked to the right.

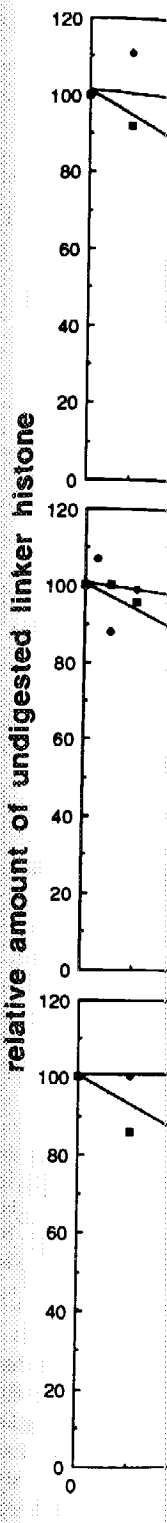
intact H1 and H5 bands (Fig. 4). Significantly, globular domains became detectable and had similar kinetics of accumulation under all three conditions. This implies that the linker histones are structured even in the absence of salt when chromatin-bound, and that the differences observed in the digestion patterns and sedimentation behavior (see below) of the three distinct fiber structures are not due to transitions in the structure of the linker histones themselves.

(c) *Chymotrypsin digestion*

The results obtained upon trypsinization of chromatin give us some insight into the accessibility of the positively charged linker histone tails in the fiber under different conformations. However, not much can be said from these studies about the globular domains, whose relative stability will make them disappear only very late during trypsin digestion, when the chromatin fiber is no longer intact. To study the accessibility of the globular domains, we have taken advantage of the fact that α-chymotrypsin preferentially cleaves adjacent to the single phenylalanine residue situated in the globular domains of both H1 and H5. This residue lies at positions 105, 108 and 109 for the different subfractions of chicken H1 (Shannon & Wells, 1987), and at position 94 in chicken erythrocyte H5 (Böhm & Crane-Robinson, 1984). Chymotrypsin digestion has the advantage that the C-terminal half of the molecule resulting from the preferential cleavage adjacent to this phenylalanine residue migrates upon SDS/polyacrylamide gel electrophoresis between the core histones and H1 and can be monitored easily (Losa *et al.*, 1984; see also Fig. 7).

Histones H1 and H5 in chromatin behaved very differently with respect to chymotrypsin digestion: while H1 was digested at a rate comparable with the rate of its digestion with trypsin, H5 was much more resistant, showing only very slow digestion in Tris, or in NaCl, and no detectable digestion in $MgCl_2$ (Figs 5 and 6). The experiments were repeated several times, performing the reaction at 10, 25 or 37 °C with the same result. Only when digestion was continued for about 30 hours at 37 °C was some slight degradation of H5 visible, the relative amount of undigested protein still being between 60 and 90% for the three conditions (the corresponding value for H1 remaining intact at this time-point was between 0 and 25%). The negative result with H5 could not be due to inactivity of the enzyme, as H1 in the same samples was readily digested. To make sure that the difference was not due to H5 being refractory to chymotrypsin digestion even when free in solution, free H1 and H5 histones were subjected to hydrolysis with immobilized chymotrypsin. The free linker histones were digested extremely efficiently, with the intact molecules disappearing within two minutes of digestion at 25 °C. To monitor properly the kinetics of digestion and the proteolytic products, the reaction with the free histones had to be performed at 10 °C and time-points had to be taken much more frequently than in the case of trypsin digestion (Fig. 7). The rate of digestion of both H1 (Fig. 7(a)) and H5 (Fig. 7(b)) was indistinguishable for the proteins dissolved in Tris, NaCl and $MgCl_2$. Furthermore, when the two proteins were digested simultaneously in an artificial equimolar mixture, H5 was digested only slightly more slowly than H1 (data not shown).

The observation that H1 digestion with chymotrypsin was independent of the conformation of the



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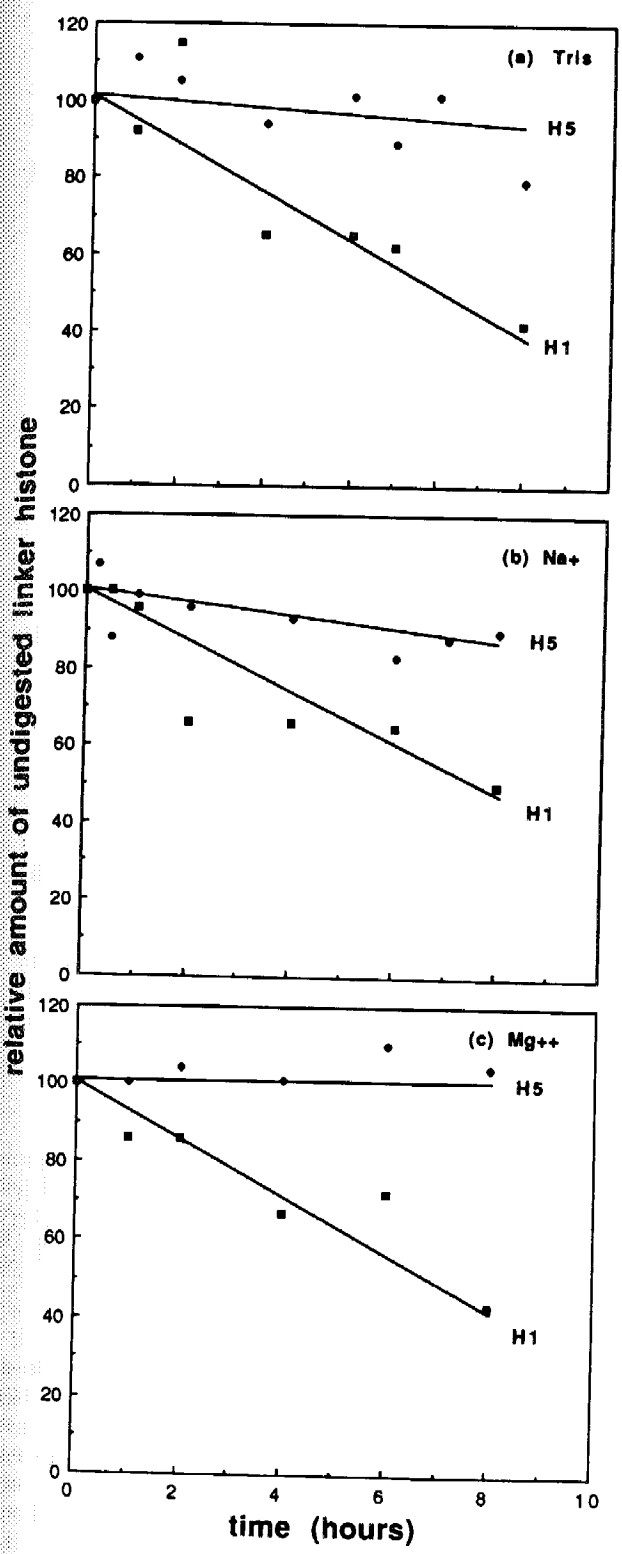

Figure 6. Quantification of the chymotrypsin digestion patterns of long chromatin, presented in Fig. 5. The electrophoretic gels were scanned and quantification was performed as described in Materials and Methods. For easier interpretation the data are presented as pairwise comparisons between histones H1 and H5 under the 3 conditions: (a) 10 mM-Tris·HCl (pH 7·5), (b) 10 mM-NaCl and (c) 0·35 mM-$MgCl_2$. The values on the ordinate represent the amount of undigested linker histone relative to the amount of histone H4, the corresponding value for the zero time-point being taken as 100%.

fiber, taken together with the ease of attack of the free histone, suggested two possibilities: either that the phenylalanine residue, which is the preferential site of attack, is not accessible even in the mononucleosome or that it becomes inaccessible upon formation of the fiber itself, independent of what particular three-dimensional conformation the fiber has adopted. To distinguish between these two possibilities, we digested isolated H1/H5-containing mononucleosomes (see Materials and Methods). The results are shown in Figures 8 and 9. Three points deserved attention. (1) H5 was more stable than H1 under all conditions. (2) Both linker histones were much more stable in Tris than in 10 mM-NaCl or 0·35 mM-$MgCl_2$. (3) In no case were digestion products seen that corresponded to the C-terminal halves of H1 and H5. The lack of C-terminal halves upon digestion of monosomes was similar to the pattern obtained upon digestion of long chromatin (see Fig. 5). This implied that the enzyme degraded the histone by attacking at sites (possibly multiple, as no discrete bands for digestion products were seen anywhere in the gel) other than the phenylalanine residue in the globular domain. The lack of attack at this position even in the nucleosome unequivocally shows that the globular domain is so situated in the nucleosome that the phenylalanine residue is buried inside the structure and is thus inaccessible to proteolytic attack by the immobilized enzyme.

(d) *Analytical centrifugation*

The issue of whether the initial cleavages introduced into the molecules of the linker histones by mild trypsin treatment already cause structural perturbations of the 30 nm fiber has been the matter of considerable controversy. Thus, Böhm & Crane-Robinson (1984) argue that a single cut in even a small fraction of the molecules might result in breakdown of the supercoil, so that subsequent cuts do not reflect the supercoil geometry. Thus, only initial rates of digestion could possibly provide insight into fiber structure. A contrary opinion is expressed by Marion *et al.* (1983*a*), who assert that the cleavage of H1 does not affect higher-order chromatin structure and that only the digestion of the terminal regions of H3 leads to unfolding of the fiber. However, in this and other studies by the latter group (Marion *et al.*, 1983*b*; Hacques *et al.*, 1990), digestion was performed at low ionic strength conditions (1 mM-sodium phosphate, 0·2 mM-EDTA, pH 7·4) in which the chromatin fiber is known to exist in an extended conformation (Thoma & Koller, 1981; Losa *et al.*, 1984; for a review, see Thoma, 1988); the positive birefringence of the undigested preparation served as the only criterion for a vaguely defined "very compact conformation", presumably the 30 nm fiber. In view of this controversy, it was important to look for possible changes in the fiber structure taking place during our mild digestion procedure. To that end,

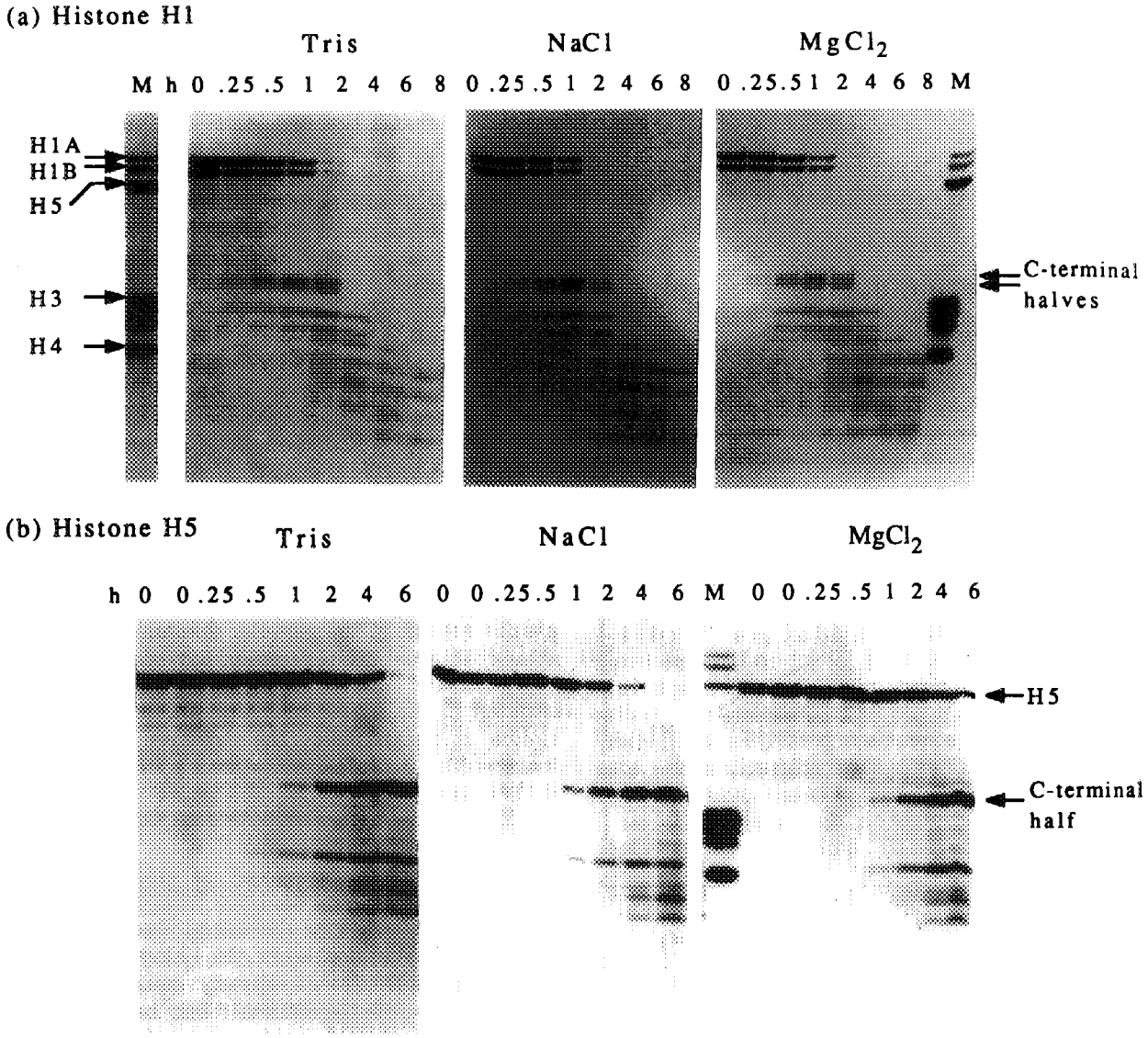

Figure 7. SDS/polyacrylamide gel electrophoretic patterns of purified histones H1 and H5 digested with chymotrypsin at 10°C. (a) Histone H1; (b) histone H5. The purified histones were dissolved in 10 mM-Tris·HCl (pH 7·5) (left panels), in 10 mM-NaCl (middle panels), or in 0·35 mM-$MgCl_2$ (right panels), respectively. The time of digestion is denoted above the lanes. Total chicken erythrocyte histones were used as markers (denoted M above the respective gels) in order to facilitate the identification of the C-terminal halves of the molecules, resulting from the preferential digestion at the sole phenylalanine residue in the globular domains (the positions of the C-terminal halves are denoted to the right).

we studied the sedimentation behavior of our initial chromatin preparation under the three conditions both before digestion, and also later, during the course of digestion, with the results presented in Figure 10.

The data are presented as integral distributions of S, calculated according to the van Holde & Weischet analysis (1978), which corrects for the effects of diffusion. As Figure 10 shows, the distribution at zero digestion time (undigested material) was relatively sharp, although the apparent sharpness is, in part at least, a consequence of concentration dependence of S. This artifact was most pronounced with the extended fibers run at low salt. The mean S value for undigested chromatin increased from about 58 in Tris to about 85 in 10 mM-NaCl (not shown) and to about 140 in 0·35 mM-$MgCl_2$, reflecting the increasing condensation of the fiber. Under all three conditions, even limited digestion produced a marked broadening of the sedimentation coefficient distribution. This was evident after only 1·5 h of digestion, when only about 10 to 20% of the linker histones had been cleaved and the core histones were still intact.

The changes in S-distribution indicated a more complex process than simple unfolding of the fiber, for a portion of the material increased in S. This might reflect either a small amount of aggregation, or a collapse of previously extended fiber regions into a more globular, condensed conformation. We favor the latter interpretation, since the increase in S was more pronounced when the molecules had been initially folded into very asymmetric 30 nm fibers.

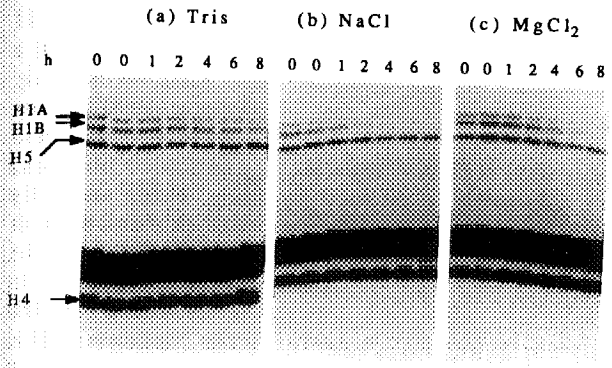

Figure 8. SDS/polyacrylamide gel electrophoretic patterns of isolated mononucleosomal particles digested with immobilized chymotrypsin. Monosomes, obtained and characterized as described in Materials and Methods, were dissolved in (a) 10 mM-Tris·HCl (pH 7·5) or in the same buffer containing (b) 10 mM-NaCl or (c) 0·35 mM-$MgCl_2$ and the digestion was carried out at 37 °C. Time of chymotrypsin treatment (h) is denoted above the lanes. The positions of H1 (see legend to Fig. 1), H5 and H4 are marked to the left.

4. Discussion

The results from these studies lead us to several important insights concerning the location of these histones in different structural forms of the chromatin fiber. In analyzing these results it is important to keep in mind that, according to the manufacturer, enzymes attached covalently to Immobilon membranes are positioned about 1·5 to 2 nm from the membrane surface. Therefore, only limited penetration into the 30 nm fiber should be possible.

(a) *Histone H5 is more protected than H1 in the chromatin fiber*

Under all conditions tested, chromatin-contained H5 was digested with immobilized trypsin more slowly than histone H1. No such difference was detected upon digestion of the free linker histones. As under our mild digestion conditions trypsin attacked only the N and C-tails of the molecules, this result implies that histone H5 and histone H1 differ in the way their tails are located in the fiber. The case of H5 being differently located in the fiber becomes especially strong from the results of chymotrypsin digestion. The almost complete resistance of fiber-contained H5 to digestion under all ionic conditions, together with its "normal" digestion in the monosome particle, implies that it is the formation of the fiber itself (the presence of adjacent nucleosomes) that makes H5 completely protected from chymotrypsin attack.

To the best of our knowledge, these results constitute the first observation that the two major linker histone types in chicken erythrocyte nuclei are located in a distinguishable manner. This difference in location might be the molecular basis for the different role of these histones in affecting transcription and replication (see Introduction). It should be noted that the stronger protection of H5 in the fiber in comparison with H1 is reminiscent of a similar differencce observed immunochemically between H1 and H1°, a histone H1 subfraction typically present only in non-dividing, differentiated cell types (Panyim & Chalkley, 1969). Banchev *et al.* (1990) reported that H1° was much less exposed to antibody binding than was H1 in mouse liver chromatin. The difference in location observed between H1, on one hand, and H5 and H1°, on the other, might be relevant to the different functions of these different H1 subtypes in chromatin of cells differing in their state of proliferation and/or differentiation.

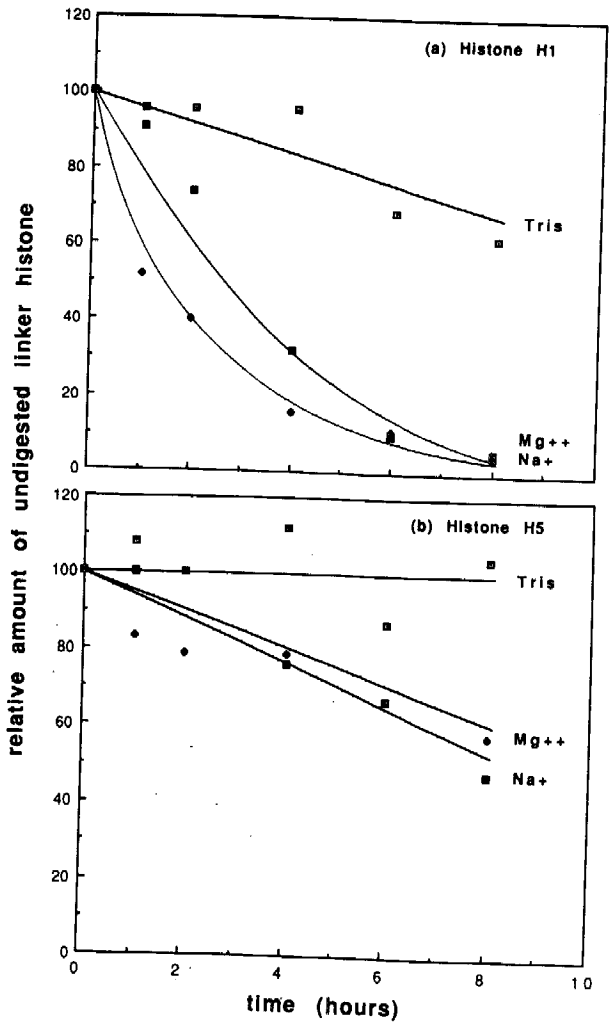

Figure 9. Quantification of the chymotrypsin digestion patterns of isolated mononucleosomal particles, presented in Fig. 8. The electrophoretic gels were scanned and quantification was performed as described in Materials and Methods. For easier interpretation the data are presented separately for each linker histone: (a) histone H1 and (b) histone H5, under the 3 conditions; 10 mM-Tris·HCl (pH 7·5), 10 mM-NaCl and 0·35 mM-$MgCl_2$ (marked on the graphs as Tris, Na^+ and Mg^{++}, respectively). The values on the ordinate represent the amount of undigested linker histone relative to the amount of histone H4, the corresponding value for the zero time-point being taken as 100%.

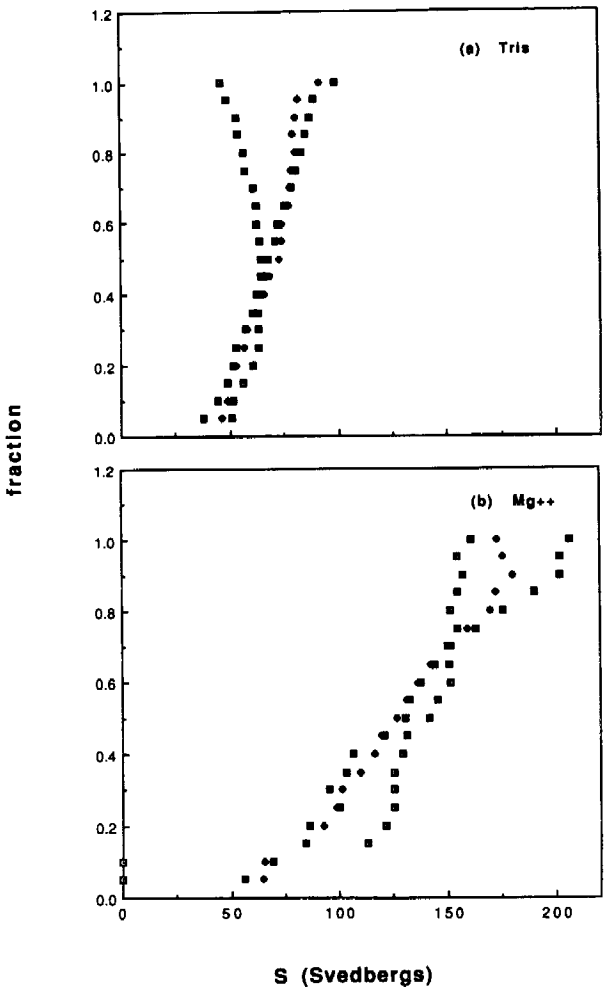

Figure 10. Integral distribution of the sedimentation coefficients of the chromatin fiber in (a) 10 mM-Tris·HCl (pH 7·5) and (b) 0·35 mM-$MgCl_2$ in the course of a trypsin digestion experiment. Digestion was at 25 °C and samples for analytical centrifugaton were withdrawn at zero time (⊡), 1·5 h (◆) and 4 (■) h. The kinetics of digestion was followed by SDS/polyacrylamide gel electrophoresis as illustrated in Figs 1 and 2.

A potentially interesting incidental observation is that in the nucleosome itself, H5 in Tris behaves differently than in Mg^{2+} or Na^{+} (Fig. 9(b)). We have no explanation at the present; clarification of this point requires additional experimentation.

(b) *N and C-terminal tails of histone H1 do not change their location significantly upon compaction of the chromatin fiber*

The data presented in Figures 1 and 2(d) show that under mild digestion conditions trypsin attacks the tails of histone H1 in a manner independent of the chromatin fiber structure. This observation implies that the location of these portions of the molecule does not change upon compaction of the fiber which, in turn, suggests that at least the majority of H1 molecules do not become internalized in the 30 nm fiber.

The issue of H1 location in chromatin fibers of different conformation has so far been studied by immunochemical approaches, with conflicting results (for a review, see Zlatanova, 1990). Some authors (Takahashi & Tashiro, 1979; Russanova *et al.*, 1987) reported that H1 in the folded fiber was not accessible to antibody binding. Using antibody populations against the intact molecule and against its globular domain, Russanova *et al.* (1987) found that the globular domain was always "hidden" in the fiber, while the tails were accessible in the extended state but protected in the condensed fiber. However, other immunological studies (Banchev *et al.*, 1990) did not detect any change in the accessibility of H1 in different fiber conformations. The antibody population used responded to antigenic determinants located both in the globular domain and in the C-tail. The results from the present study are in accordance with those of Banchev *et al.* (1990), despite the entirely different approaches used in the two studies.

(c) *The tails of histone H5 become partially inaccessible (internalized) in the 30 nm fiber*

One of the major findings of this work is that upon formation of the 30 nm fiber, the accessibility of the terminal portions of the H5 molecules to immobilized trypsin decreases. Significantly, this behavior of histone H5 differs from that of H1, the accessibility of whose N and C-tails does not change upon fiber condensation. The protection from digestion is not complete, which implies one of two things: (1) either all H5 molecules become somewhat less accessible due to steric hindrance by chromatin components becoming tightly packed upon condensation; or (2) that some of the molecules become internalized in the fiber, while others remain on the outside. To discriminate between these two possibilities would require other approaches. To our knowledge, these data are the first in the literature to provide information on the behavior of the H5 tails in condensation. Most immunochemical studies concerning histone H5 have made use of antibodies directed against epitopes in the immunodominant globular domain (see Zlatanova, 1990).

(d) *Phenylalanine in the globular domains of H1 and H5 is buried inside the structure both at the level of the nucleosome and in the fiber*

As trypsin digestion could give insights only into the location of the terminal domains of the linker histone, the study was complemented by the use of immobilized chymotrypsin, which preferentially attacks adjacent to the single phenylalanine residue of the globular domain. At all conformations of the fiber and in the isolated H1/H5-containing nucleosomes, this amino acid was completely inaccessible to the enzyme and no attack was observed even at 37 °C and at prolonged digestion times. Although

H1 was attacked by chymotrypsin, cleavage was not adjacent to the phenylalanine residue in the globular domain, since no C-terminal halves of the molecules were observed in the gel: The cleavage evidently involved multiple other amino acid residues situated away from the phenylalanine residue. These results imply that the globular domain both in the nucleosome and in the fiber is situated in such a way that its phenylalanine residue is inaccessible to the immobilized enzyme. An alternative explanation for the lack of the C-terminal half could be that other sites become equally accessible to the enzyme when the linker histone is bound to the nucleosome or to the fiber. This possibility seems to us less likely, as it would require drastic changes in the specificity of the enzyme or in the conformation of the protein.

The observation that the accessibility of the phenylalanine residue in the globular domain was not a function of the fiber conformation differed from the results reported by Losa *et al.* (1984). The explanation for this discrepancy might be connected to the fact that soluble chymotrypsin was used in that study. The length of the cross-link between the enzyme and the membrane in our study (15 to 20 Å) would severely restrict its action to molecules in its immediate vicinity. A possible alternative explanation that the difference is due to the fact that rat liver chromatin contains only H1 as opposed to the presence of both H1 and H5 in chicken erythrocytes seems to us less likely.

The question of the location of the globular domain of histone H5 in the extended and condensed fiber has been earlier addressed in immunochemical studies, with conflicting results. Dimitrov *et al.* (1987) showed that antibodies to the globular domain of H5, made large enough by cross-linking to the bulky ferritin molecule so as not to penetrate into the interior of the fiber, reacted with chromatin at salt concentrations only up to about 20 mM-NaCl; at higher ionic strength no reaction was observed. These data were interpreted as indicating that the globular domain of H5 becomes internalized in the 30 nm fiber. While the data seem reasonably solid, the interpretation can be questioned for two reasons. (1) The chromatin fiber was fixed with glutaraldehyde, which might have led to artifactual hiding of some of the antigenic determinants, as demonstrated more recently (Thibodeau & Ruiz-Carrillo, 1988). It is possible that in the condensed fiber, the glutaraldehyde fixation might lead to even heavier crosslinking and hence to more extensive hiding of antigenic determinants. (2) The immunochemical reaction with the fiber gradually lessened with increasing the ionic strength and was already negligible at 20 to 30 mM-NaCl, a salt concentration where the fiber is still a closed zig-zag. This early disappearance of reactivity might be a consequence of the relatively low initial intensity of the reaction but the data do not allow unambiguous interpretation. Contrary to the conclusions reached by Dimitrov *et al.* (1987), Thibodeau & Ruiz-Carrillo (1988) assert that the globular regions of H5 is equally accessible in the extended and the condensed fiber.

Our results demonstrate that chymotrypsin must be used with caution as a globular domain probe for studies of higher-order structure, since the preferentially cleaved, globular domain-contained phenylalanine residue is inaccessible even in the nucleosome. It is clear that alternative probes, including antibodies of high reactivity, possibly immobilized to create large but sensitive probes, should be sought to resolve this issue.

(e) *Linker histone digestion and ordered chromatin structure*

The sedimentation velocity experiments carried out in this work show that the integrity of almost all of the linker histone molecules seems to be required for the proper maintenance of ordered chromatin structure. It is of importance that most of the change we observe in the distribution of S occurs early during the digestion (1·5 h), when only a small fraction of H1 or H5 has been cleaved; the distribution at four hours shows little further change. Our results are in much better agreement with the position of Böhm & Crane-Robinson (1984) than with that of Hacques *et al.* (1990).

There are two possible explanations for the effect of linker histone cleavage on chromatin structure. First, the changes in the fiber structure that we observe upon digestion may be caused by dissociation of the globular domain from the fiber and thus the initial cleavages in the histone molecules might be accompanied by complete loss of individual H1 or H5 molecules from the fiber. Alternatively, if the globular domain remains bound to chromatin after initial cleavages in the linker histone molecules, the changes in the fiber structure might reflect the inability of the globular domain by itself to maintain long-range interactions required to form higher-order structures, as proposed earlier by Allan *et al.* (1986).

Our experimental approach does not allow us to determine whether the globular domain remains bound to chromatin after digestion of the tails. Such a discrimination would require separation of the unattached digestion products from the long chromatin fiber. Alternatively, the differences in the behavior of the globular domain when the protein is free in solution or attached in chromatin with respect to chymotrypsin, could be used to approach this question. This would require consecutive trypsin and chymotrypsin treatments under carefully controlled conditions.

The sedimentation velocity measurements performed during the course of trypsin digestion suggest that linker histones are important in maintenance not only of the higher order structure of the chromatin fiber, as accepted hitherto, but of ordered fiber structure in general, independent of its particular condensation state. Indeed, the broadening of the distribution of the sedimentation coefficient with time of digestion, which reflects changes in the

fiber structure, is observed not only in 0·35 mM-$MgCl_2$, but also in Tris and in 10 mM-NaCl. This observation together with the known fact that the linker histones are involved in the structuring of the nucleosomal particle itself (Allan *et al.*, 1980) points to the importance of this histone class at all levels of chromatin organization.

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