Contributions of Linker Histones and Histone H3 to Chromatin Structure: Scanning Force Microscopy Studies on Trypsinized Fibers

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ABSTRACT Little is known about the mechanisms that organize linear arrays of nucleosomes into the three-dimensional structures of extended and condensed chromatin fibers. We have earlier defined, from scanning force microscopy (SFM) and mathematical modeling, a set of simple structural determinants of extended fiber morphology, the critical parameters being the entry-exit angle between consecutive linkers and linker length. Here we study the contributions of the structural domains of the linker histones (LHs) and of the N-terminus of histone H3 to extended fiber morphology by SFM imaging of progressively trypsinized chromatin fibers. We find that cleavage of LH tails is associated with a lengthening of the internucleosomal center-to-center distance, and that the somewhat later cleavage of the N-terminus of histone H3 is associated with a flattening of the fiber. The persistence of the "zigzag" fiber morphology, even at the latest stages of trypsin digestion, can be attributed to the retention of the globular domain of LH in the fiber.

INTRODUCTION

Much is now known concerning the structure of the nucleosome, the fundamental repeating unit of eukaryotic chromatin (van Holde, 1988; Tsanev et al., 1992). In the core particle of the nucleosome, 146 bp of DNA is wrapped in ~1.65 left-handed superhelical turns around an octameric complex consisting of two molecules of each of the core histones H2A, H2B, H3, and H4 (Luger et al., 1997). Successive core particles are connected by stretches of linker DNA to which members of the fifth major histone class, the so-called lysine-rich or linker histones, are bound. How this linear array of nucleosomal particles is organized in the nucleus remains an enigma. During the first decade after the discovery of the nucleosome, the literature was flooded with specific models attempting to describe the so-called 30-nm fiber structure observed at physiological salt concentrations (for a review, see van Holde, 1988; Tsanev et al., 1992). Almost all proposed models for the "30-nm fiber" have been based on the idea of regularity of structure. We have recently shown that the fiber has a very similar diameter, albeit a more extended structure, even under low-salt conditions (Leuba et al., 1994a). We (Leuba et al., 1994a; Yang et al., 1994; van Holde and Zlatanova, 1995) and others (Woodcock et al., 1993; Horowitz et al., 1994; Woodcock and Horowitz, 1995) have presented evidence suggesting that the fiber has only limited regions of regularity. We have suggested that the term "30-nm fiber" be abandoned as a designator for the more condensed, high-salt structure and that the latter should be called the "condensed" or "compact" fiber (van Holde and Zlatanova, 1995), to distinguish it from the more "extended" fiber observed at lower ionic strength. In terms of biological relevance, studies of the structures of the more extended forms of the chromatin fiber—the kinds of conformation needed for "reading" processes—may be of greater importance than understanding the structure of the inert, condensed fiber.

Not only are the exact morphologies of the extended and condensed fibers uncertain, but very little is known about the molecular mechanisms that organize the linear array of nucleosomes into three-dimensional fibers. Our previous work used the newly developed technique of scanning force microscopy (SFM). This technique allows imaging of biological macromolecules and structures in ambient conditions, without staining or shadowing, in air or under liquids (Bustamante et al., 1993, 1994; Bustamante and Keller, 1995). From SFM images of native and linker histone-depleted chromatin fibers in air and mathematical simulations of such fibers, we proposed a set of structural parameters that determine extended fiber morphology. Two of these parameters—the entry-exit angle of DNA in and out of the core particle, and the variable length of the DNA linker—have also been recognized as determinants of fiber morphology from modeling studies combined with electron microscopy (EM) (Woodcock et al., 1993). Moreover, we further suggested that a major role of linker histones is to maintain the entry-exit angle (Yang et al., 1994), consistent with the presumed location of its globular domain at or near the entry-exit site of the DNA at the nucleosome (Boulikas et al., 1980; Allan et al., 1980).

Several questions regarding the molecular determinants of chromatin fiber structure remain. In particular, is it the

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central globular domain of the linker histones, or their less organized basic N- and C-terminal tails (reviewed in Zlatanova and van Holde, 1996), or both that are responsible for maintaining the entry-exit angle? What is the role, if any, of the core histone tails in the three-dimensional organization of the low-ionic-strength fiber? The core histone tails do not participate in the structure of the core particle, but rather protrude (van Holde, 1988; Luger et al., 1997). These tails have been implicated in promoting nucleosome-nucleosome interactions, and have been shown to interact with linker DNA and linker histones (for further discussion and references, see van Holde and Zlatanova, 1996). If they were fully extended, some of the tails could span rather long distances, creating opportunities for interactions with other components of the fiber. Moreover, core histone tails seem to be involved in the salt-induced compaction of the extended, low-ionic-strength fiber. In fact, older papers report changes in physical parameters of the condensed chromatin fiber upon proteolytic removal of these tails (Chatterjee and Walker, 1973; Saccone et al., 1983; Marion et al., 1983a,b; Makarov et al., 1984; Hacques et al., 1990). Allan et al. (1982) have shown that polynucleosomes that contain core histones devoid of the tails remain substantially uncondensed in high salt, even in the presence of histone H1. Similar findings were reported for reconstituted nucleosomal arrays in the absence of histone H1 (Garcia-Ramirez et al., 1992; Fletcher and Hansen, 1995; Schwarz et al., 1996).

We have approached these questions by combining the imaging capabilities of SFM with trypsinization of isolated long chromatin fibers under extremely mild conditions, using immobilized trypsin. The fiber structures were imaged in an SFM operated in the tapping mode (Leuba et al., 1994a). These images were then quantitatively characterized by extensive measurements of internucleosomal center-to-center distances, fiber heights, and the angles formed by lines connecting three successive nucleosomal centers. These will hereafter be referred to as internucleosomal angles; they are obviously closely related to, but not identical to, the entry/exit angles between successive linkers. SFM imaging allows us to obtain such structural data at the single-fiber level, a great advantage over the population-average information provided by other physical methods.

We find that 1) cleavage of linker histone (LH) is associated with a lengthening in the internucleosomal center-to-center distance, 2) the somewhat later cleavage of H3 tails correlates with an apparent loss of the three-dimensional organization of the fibers, and 3) with core histones H4, H2B, and H2A intact, and the globular domain of LH still present, the fibers still maintain a zigzag organization.

MATERIALS AND METHODS

Preparation and fixation of chromatin

Chicken erythrocyte chromatin was prepared as in Yager et al. (1989), with a decrease in the amount of micrococcal nuclease used to allow isolation of longer fibers (Leuba et al., 1994b). Soluble chromatin was dialyzed versus 5 mM triethanolamine-HCl (TEA-HCl) (pH 7.0) and stored on ice. For SFM imaging, chromatin was fixed in 0.1% glutaraldehyde overnight (Thoma et al., 1979).

Trypsin hydrolysis

Chromatin fibers were hydrolyzed by immobilized trypsin (Sigma type XIII) as described by Zlatanova et al. (1995) and Leuba et al. (1993). Aliquots were removed at the indicated times and analyzed by both sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and SFM. Mock-digested fibers (no membrane with immobilized trypsin present in the chromatin solution) did not show detectable histone degradation, even after 24 h of incubation.

To determine whether trypsinized LH globular domain remained bound to chromatin, the trypsinized sample was placed in a Centricon 100 tube (Amicon, Beverly, MA) and centrifuged for 30 min at 1000 $\times$ g. Two milliliters of 5 mM triethanolamine (pH 7.0) was added and Centricon-centrifuged for 30 min. The washing step was repeated three times before the high-molecular-weight chromatin was resuspended in 0.5 ml of buffer, and analyzed by SDS-PAGE. Control Centricon 100 tubes containing only pure LHs were run in parallel.

Scanning force microscopy and quantitation of images

Twenty microliters of glutaraldehyde-fixed chromatin was deposited for 1 min on a freshly cleaved mica surface. The mica was rinsed with 10 drops of Nanopure (Barnstead, Dubuque, IA) water, and the visible liquid was removed by a 15-s flow of nitrogen gas. Tapping-mode SFM (Digital Instruments, Santa Barbara, CA) was performed as in Leuba et al. (1994a). SFM images were transferred to ALEX, an image analysis program written by Mark Young and Claudio Rivetti in Matlab (MathWorks, Natick, MA) for use on the Indigo workstation (Silicon Graphics, Mountain View, CA). (ALEX source code can be obtained via ftp through the internet at alice.uoregon.edu.) The three-dimensional coordinates of the center of the top of each nucleosome in the fiber were determined, following the path of the fiber. When the order of successive nucleosomes was not clear, the shortest path was chosen. Independent measurements performed on different days on the same set of images gave practically identical results. From the three-dimensional coordinates it was straightforward to determine the heights of nucleosomes in the fibers, the center-to-center distances of successive nucleosomes, and the angles formed by lines connecting the centers of three successive nucleosomes in the fibers. Given coordinates (X_1, Y_1, Z_1) and (X_2, Y_2, Z_2) of two adjacent nucleosomes, the center-to-center distance was calculated by

$$D_{1,2} = \sqrt{[(X_1 - X_2)^2 + (Y_1 - Y_2)^2 + (Z_1 - Z_2)^2]}$$

Given coordinates (X_1, Y_1, Z_1), (X_2, Y_2, Z_2), (X_3, Y_3, Z_3) of three sequential nucleosomes, the internucleosomal angle ($\theta_{1,2,3}$) was calculated from the law of cosines:

$$\cos(\theta_{1,2,3}) = \frac{[(D_{1,\ 2})^2 + (D_{2,3})^2 - (D_{1,3})^2]}{(2D_{1,2}D_{2,3})}$$

The maximum angle measured was 180°; for example, 90° and 270° were both taken to be 90°. Center-to-center distances, heights, and angles were also measured with the original software of the scanning force microscope, with results similar to the ones determined in ALEX (data not shown).

RESULTS

SFM imaging and measurements

SFM uses a sharp tip at the end of a flexible cantilever to scan and sense the topography of a sample deposited on a

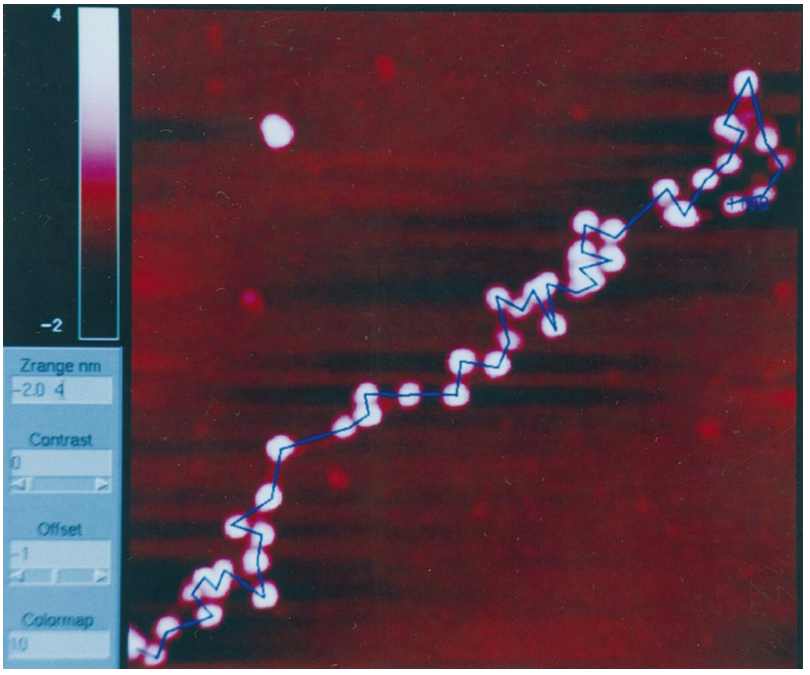

FIGURE 1 Scheme illustrating the measurements performed on experimental SFM images. Centers of consecutive nucleosomes in a fiber are marked by consecutive computer mouse clicks which record both *X*, *Y* position and height (*Z*) of the top of the nucleosomal image. The three-dimensional coordinates of these centers are used to determine center-to-center distances, angles formed by lines connecting the centers of three successive nucleosomes, and fiber heights according to the formulae given in the text. Image is 500 nm × 500 nm in size. Heights are indicated by varying shades of color, with low regions in reddish purple and higher regions in increasingly lighter tones of reddish purple, in a range of 0–15 nm.

flat surface (Binnig et al., 1986), usually mica or glass. In the tapping mode of operation, the cantilever is oscillated near its resonance frequency as the sample is scanned laterally (Bustamante and Keller, 1995). The effect of the sample is to reduce the amplitude of oscillation. This signal is used in feedback to keep the reduction in amplitude constant during scanning. Tapping mode operation decreases the pressure on the sample and virtually eliminates shear forces, thus reducing the molecular motion and deformation that often occur in contact mode operation.

As mentioned above, SFM has several important advantages over more classical imaging techniques, like EM. Even when imaging is performed in air, at room temperature and humidity, the sample preserves a layer of liquid water (Grigg et al., 1992; Hu et al., 1995); thus extensive damage due to drying is avoided.

SFM imaging can be performed on unfixed, native chromatin fibers (Leuba et al., 1994a). For the purposes of the present work, however, we used glutaraldehyde-fixed material, which consistently showed higher image quality and reproducibility than unfixed fibers (see Leuba et al., 1994a, for a comparison of SFM images of unfixed and fixed chromatin fibers). Fixation may also decrease sample compliance and reduce tip-induced deformation and deposition artifacts, which may be different in differently prepared unfixed fibers. This is an important criterion when one is attempting to compare morphologies of macromolecular complexes, for it is conceivable that differences in stability upon deposition could be incorrectly interpreted as differences in solution conformation. Glutaraldehyde fixation seemed the method of choice for maximally preserving the solution structure as it exists during the fixation step (Thoma et al., 1979; De Murcia and Koller, 1981; Russanova et al., 1987; Leuba et al., 1994a).

A number of control experiments were performed to evaluate the effect of fixation. First, when isolated di- or trinucleosomal particles were imaged, no morphological difference was seen between native and fixed material (data not shown). Moreover, actual measurements of the three structural parameters in the trinucleosomes—internucleosomal distances, angles, and heights—did not indicate any statistically significant differences between fixed and unfixed particles. Finally, when long chromatin fibers were fixed and imaged at low ionic strength, the only change observed as a result of fixation was a slight compaction,

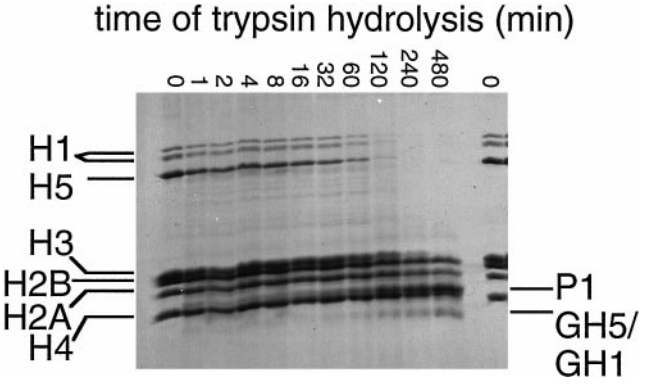

FIGURE 2 Example of SDS-PAGE of histone products of trypsin hydrolysis of chicken erythrocyte chromatin fibers. Trypsin hydrolysis was performed as described (see Materials and Methods). Aliquots of the hydrolyzed fibers were examined at the time points indicated in the figure. The positions of intact histones are marked on the side of the marker lane containing undigested chromatin. The main product of digestion of histone H3, produced by trypsin cleavage at amino acid residue 20, migrates just above histone H4 and is marked as P1 (Böhm and Crane-Robinson, 1984); the products of digestion of histones H1 and H5 are marked as GH1/GH5.

expressed as somewhat shorter internucleosomal distances, and somewhat greater heights (see also below). However, these differences were generally much smaller than the structural changes observed in the actual experiments, and therefore did not complicate the interpretation of the results.

The inferences from the appearance of the different structures studied were corroborated by measurements and statistical analysis of fiber parameters (Fig. 1). The center of each visible nucleosome in the fiber was assigned *X*, *Y*, and *Z* coordinates, and these were used as input data in software algorithms to produce values for the structural parameters (see Materials and Methods). Two points are to be kept in mind in evaluating the data. First, by necessity, the measurements took into account only those nucleosomes that are visible on the upper portion of the folded fibers, and it was difficult, in some cases, to decide upon the exact order of nucleosomes in a fiber. Neither of these problems is of concern in flat, extended structures, where nucleosomes are well separated and exposed to the scanning tip, but may affect the quantitative analysis of more three-dimensional fibers. However, we emphasize that it is the change in parameters, rather than their absolute values, that is of primary concern in this study. Moreover, if any subjective selection of somewhat more extended fibers took place during the data analysis, it would have tended to underestimate rather than overestimate the differences between flat and three-dimensional structures.

The second point concerns the physical meaning of the measured parameters. The center-to-center distance between nucleosomes is not expected to be affected by the broadening effect of the tip (Bustamante et al., 1994), and can be used to describe the degree of extension of the fibers. The measured angles (defined as the angle between lines connecting centers of three successive nucleosomes; see also Materials and Methods) can also be used to characterize the extension of the fibers and the restraints upon linker DNA trajectories. Finally, fiber heights are only significant on a comparative basis, because tapping-mode SFM imag-

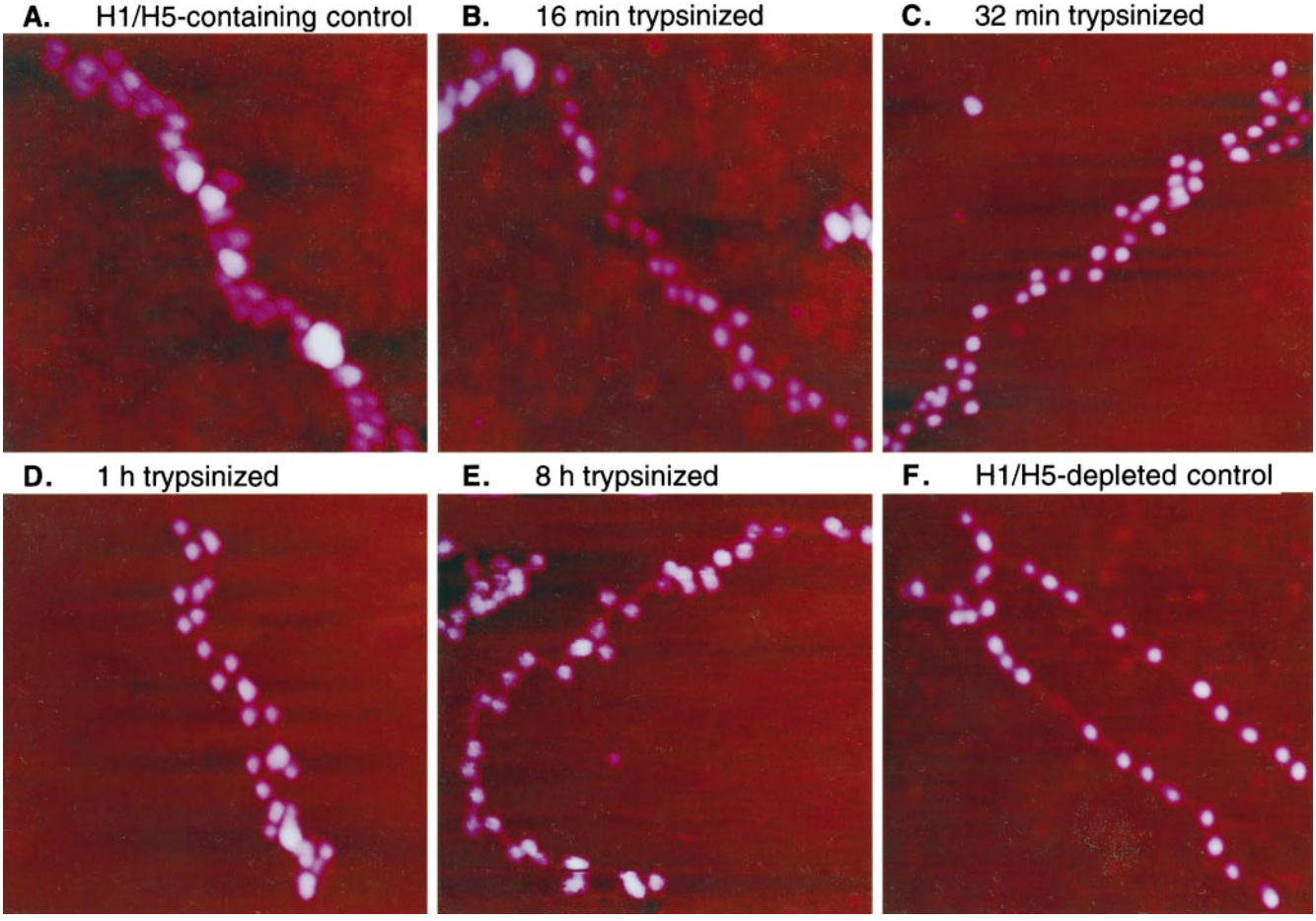

FIGURE 3 SFM images of mildly trypsinized chicken erythrocyte chromatin fibers. (*B–E*) SFM images of chromatin fibers that are trypsinized for the amount of time shown, and then glutaraldehyde-fixed. Images of untrypsinized control (*A*) and linker histone-stripped fibers (*F*) are shown for comparison. Imaging was performed in air at ambient humidity and temperature on samples deposited on mica. The differences in the apparent diameter of nucleosomes in different images are due to the differences in the radii of curvature of the particular scanning tips that were used for imaging. This does not affect the *X*, *Y*, *Z* coordinates of the nucleosomal centers. All images are 500 nm × 500 nm in size. Heights are indicated by varying shades of color with low regions in dark reddish purple and higher regions in increasingly lighter tones of reddish purple, in the ranges of 0–15 nm (*A–D*) and 0–7.5 nm (*E, F*).

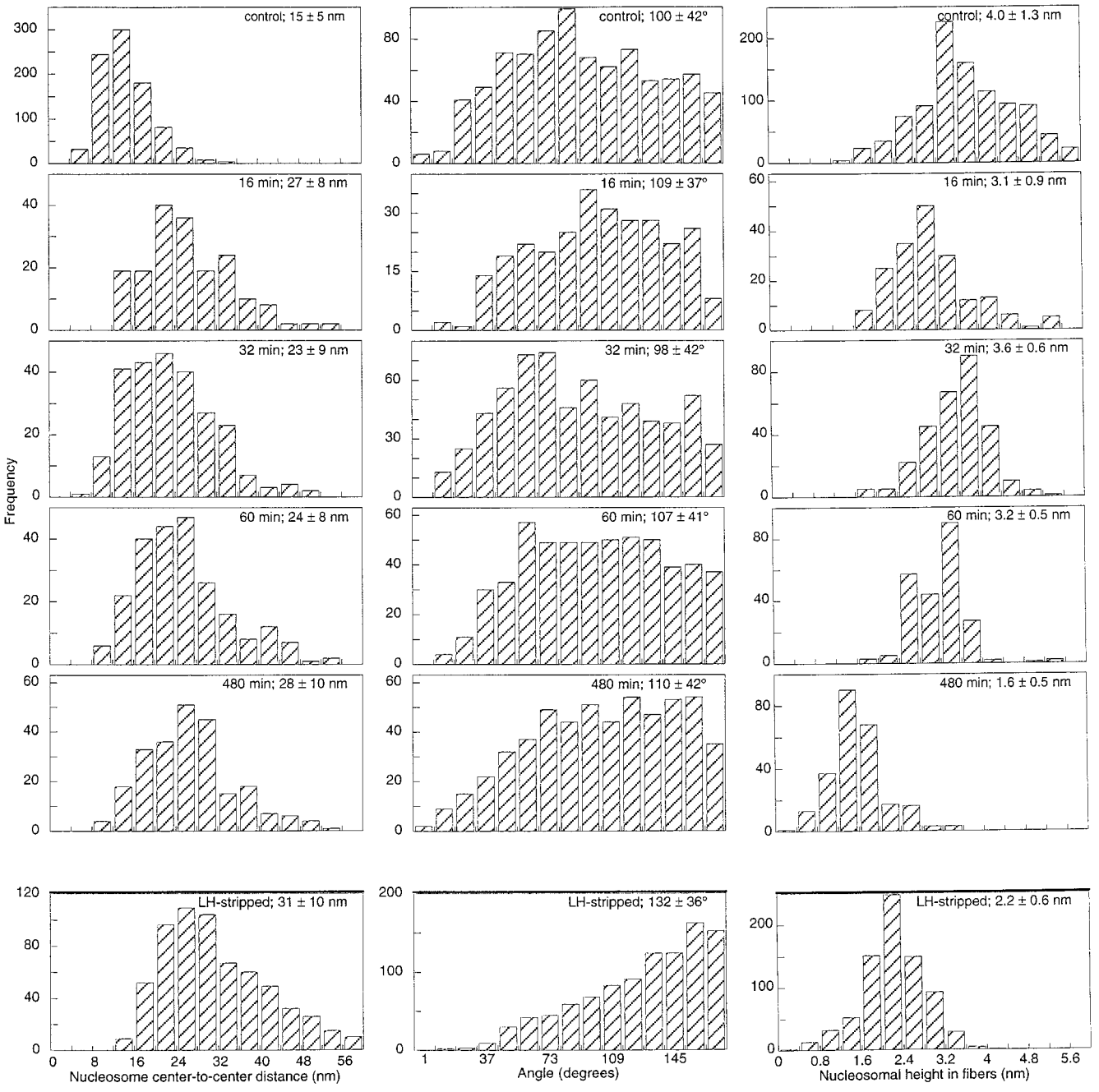

FIGURE 4 Frequency distribution histograms of center-to-center internucleosomal distances (*left-hand column*), internucleosomal angles (*center column*), and fiber heights (*right-hand column*) as a function of time of trypsin digestion, as denoted on each panel. The topmost panels in all columns show the distributions obtained on control undigested fibers, and the bottommost panels show the respective distribution histograms for LH-depleted chromatin fibers. The numbers in each panel indicate the average values for the measured parameters with standard deviations. Note that the center-to-center distance for the control fiber is ~15 nm instead of ~20 nm, as expected on the basis of biochemically determined average linker length in chicken erythrocyte chromatin. The smaller than expected value is due to glutaraldehyde fixation, because similar measurements performed on unfixed fibers gave a value of ~20 nm (Zlatanova et al., 1998). For further details, see Materials and Methods.

ing in air tends to decrease actual heights by a factor that depends on adhesion (van Noort et al., 1997). For purified mononucleosomal particles, the flattening factor is ~2, because SFM estimations give a height of ~2.5–3 nm, instead of the 5.5 nm expected on the basis of crystallography data (Richmond et al., 1984; Luger et al., 1997).

Trypsin hydrolysis of chromatin fibers leads to progressive loss of three-dimensionality

Mild trypsin hydrolysis in chromatin fibers leads to degradation of LH tails, closely followed by degradation of the H3 N-terminus (Fig. 2; see also Zlatanova et al., 1995). This

is in agreement with numerous previous reports (e.g., Chatterjee and Walker, 1973; Weintraub and van Lente, 1974). In this paper, we examine the effect of this degradation on fiber morphology at low ionic strength, using SFM. Examples of SFM images of undigested chromatin fibers and fibers at different points of hydrolysis are shown in Fig. 3, with variation in color tones displaying the apparent heights of different nucleosomes above the mica surface. The nonhydrolyzed control fibers (Fig. 3 *A*) exhibited a three-dimensional arrangement of nucleosomes, as observed previously (Leuba et al., 1994a). Fibers at intermediate stages of hydrolysis had partially lost the three-dimensional nucleosomal organization; the unraveled stretches of such fibers displayed a zigzag arrangement of successive nucleosomes (Fig. 3, *B–E*). Note that the zigzag mutual disposition of nucleosomes is still present at the end point of digestion, when the tails of both the LHs and H3 had been cleaved (see Fig. 3 *E*). Control experiments indicated that the remaining core histones were intact and that the globular domain of the LHs was still attached to these partially hydrolyzed fibers (see Materials and Methods). On the other hand, fibers totally stripped of LHs lost this zigzag character (Fig. 3 *F*).

The unraveling of the chromatin fiber structure upon trypsin hydrolysis could be quantitatively followed by measuring center-to-center distances, internucleosomal angles, and apparent fiber heights in SFM images. Frequency distribution histograms of center-to-center distances show that trypsin hydrolysis is accompanied by a gradual increase in this parameter from the ~15 nm for the undigested controls to ~28 nm at 8 h of digestion (Fig. 4, *left-hand column*). For comparison, the average center-to-center distance for LH-stripped fibers is ~31 nm. Similar distribution histograms for fiber apparent heights show a progressive decrease from the ~4-nm value for the control fiber to ~1.6 nm at the end point of trypsin digestion (Fig. 4, *middle column*). This value is similar to that of LH-stripped fibers. In marked contrast to these results, the distribution of internucleosomal angles remains virtually unchanged during the digestion (Fig. 4, *middle column*). In particular, it never approaches the skewed distribution found with LH-stripped chromatin fibers.

Plotting these image parameters and the extent of histone digestion as a function of digestion time revealed that the change in internucleosomal distances correlated best with the digestion of the LHs, whereas the change in fiber heights paralleled more closely the later cleavage of histone H3 N-terminus (Fig. 5). Note that the mean angle did not change throughout the experiment.

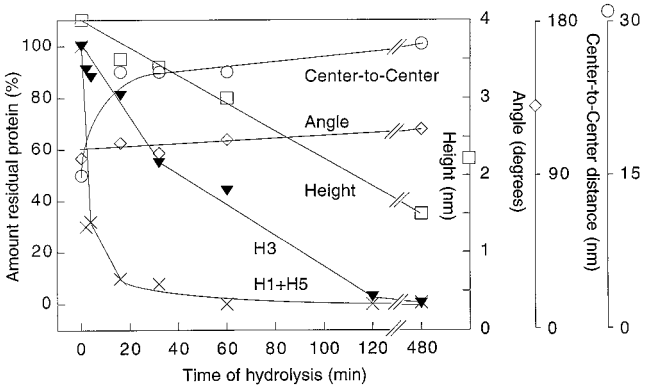

FIGURE 5 Kinetics of digestion of linker histones and histone H3, as measured by SDS gel electrophoresis of histones. The concomitant changes in fiber parameters are plotted on the same time scale to facilitate interpretation of the results. ×, Amount of residual linker histone; ▼, amount of residual core histone H3; ○, average center-to-center distances; ◇, average internucleosomal angles; □, average heights of nucleosomes within the fibers. □, ◇, ○ on the respective *Y* axes: average heights, angles, and center-to-center distances of control linker histone-stripped chromatin fibers.

DISCUSSION

Understanding the structure of the extended chromatin fiber at low ionic strength is of crucial importance, because extended fibers presumably represent the form in which the DNA template is presented to cellular machineries conducting transcription, replication, and repair (van Holde and Zlatanova, 1996). To gain further insight into the molecular determinants that define the three-dimensional structure of extended chromatin fibers, we have combined biochemical techniques and SFM. Despite its many advantages, it must be understood that there are limitations to this imaging technique. SFM is a surface method, and sample attachment to the surface may lead to some alterations of the solution structure. In particular, the flattening observed with some structures may be the result of such effects. Nevertheless, the very fact that differences in fiber composition can yield either flat or three-dimensionally organized morphology on the same kind of surface clearly points to intrinsic differences in the solution structures and must reflect differential structure or stability as a function of their histone composition, because all other experimental conditions have been kept the same.

The main conclusion from this work is that the globular domain of the linker histones, the linker histone tails, and the N-terminal tail of core histone H3 all affect chromatin fiber structure, yet in subtly different ways. The globular domain of linker histones helps fix the angle between DNA duplexes entering and exiting the nucleosome, as evidenced by the observation that its presence in the digested fiber is necessary and sufficient to preserve the zigzag morphology of the fiber and to keep the distributions of internucleosomal angles constant during digestion. On the other hand, the tails of the LH contribute primarily to the close packing of adjacent nucleosomes, as evidenced by the increase in internucleosomal distances that accompanies cleavage of these tails. The N-terminal tails of H3 appear to contribute mainly to the stability of the three-dimensional fiber structure, because their cleavage leads to a dramatic flattening of fibers.

Although the experiments described herein point very strongly to these conclusions, we remained concerned by certain aspects. Because the digestion of LH tails and that of

H3 tails overlap to a certain extent, the separation of their functions was not so clearly defined as we would have liked. Furthermore, it is difficult to tell, in such experiments, whether the cleaved fragments of the histone tails are still retained in the chromatin fiber. To surmount these difficulties, we have devised another approach to more critically evaluate the conclusions derived from this work. This is described in the following paper.

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