Linker DNA Bending Induced by the Core Histones of Chromatin†

J. Yao,‡,§ P. T. Lowary,‡,∥,⊥ and J. Widom*,‡,§,∥,⊥,#

Departments of Chemistry, Biochemistry, and Biophysics and Beckman Institute, University of Illinois, Urbana, Illinois 61801

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ABSTRACT: We have previously reported that ionic conditions that stabilize the folding of long chromatin into 30-nm filaments cause linker DNA to bend, bringing the two nucleosomes of a dinucleosome into contact [Yao, J., Lowary, P. T., & Widom, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7603–7607]. Dinucleosomes are studied because they allow the unambiguous detection of linker DNA bending through measurement of their nucleosome–nucleosome distance. Because of the large resistance of DNA to bending, the observed compaction must be facilitated by the hitsones. We have now tested the role of histone H1 (and its variant, H5) in this process. We find that dinucleosomes from which the H1 and H5 have been removed are able to compact to the same extent as native dinucleosomes; the transition is shifted to higher salt concentrations. We conclude that histone H1 is not essential for compacting the chromatin filament. However, H1 contributes to the free energy of compaction, and so it may select a single, ordered, compact state (the 30-nm filament, in long chromatin) from a family of compact states which are possible in its absence.

The lowest level of structure of a chromosome is a linear array of nucleosomes connected by linker DNA. In the next level of structure, this nucleosome filament is compacted into a shorter, wider fiber having a diameter of ~30 nm. Studies from many laboratories have led to helical models of the 30-nm fiber, in which nucleosomes (which have the shape of disks, with a diameter of 11 nm and a height of 6 nm; Richmond et al., 1984) are packed edge-to-edge parallel to the 30-nm fiber axis and radially around it, with their disk faces roughly parallel to the axis [for reviews, see Felsenfeld and McGhee (1986) van Holde (1989) and Widom (1989)]. However, questions remain about how these helically arranged nucleosomes are connected spatially by DNA. For example, it is not known whether consecutive nucleosomes along the DNA molecule are also consecutive on the superhelix of the 30-nm fiber, nor is it known whether the linker DNA is bent or kept straight in the 30-nm fiber. Other related important questions include the following: What interactions are responsible for folding the nucleosome filament into the 30-nm fiber? What is the mechanism by which cells exert local control over the level of folding of their chromosomes?

In a previous study (Yao et al., 1990), we showed that, in conditions in which long chromatin folds in vitro into 30-nm fibers, linker DNA in dinucleosomes bends to bring the two (consecutive) nucleosomes into spatial contact. This bending of linker DNA is required by the widely accepted solenoid model of the 30-nm chromatin fiber (Finch & Klug, 1976; Thoma et al., 1979; Widom & Klug, 1985). In that model, the nucleosome filament is arranged in a one-start helix; consecutive nucleosomes are lateral neighbors and are brought into direct contact in the structure despite being separated (in the one-dimensional structure) by the linker DNA.

We wish to determine what interactions are responsible for bringing consecutive nucleosomes together and bending the intervening linker DNA. Histone H1 is known to be required for the proper formation of 30-nm filaments [for reviews, see van Holde (1989) and Widom (1989)], but several lines of evidence suggest that core histones too are involved in extranucleosomal interactions and possibly contribute to the higher order structure. Although it fails to fold into ordered 30-nm fibers (Finch & Klug, 1976; Thoma et al., 1979; Widom, 1989; Fujiwara et al., 1989), chromatin that has been stripped of H1 and H5 still undergoes some compaction when titrated with increasing $[M^+]$[1] (Noll & Kornberg, 1977; Spadafora et al., 1979; Butler & Thomas, 1980; Hansen et al., 1989; Fujiwara et al., 1989). Nucleosome core particles (which lack H1 and H5) aggregate in relatively mild conditions into ordered arrays (Dubochet & Noll, 1978). On a DNA molecule that is long enough to bind two or more histone octamers (in two or more nucleosomes), the presence of one octamer influences the location of successive ones in a manner reflecting attractive nucleosome–nucleosome interactions and not simply (repulsive) steric exclusion (Drew & Calladine, 1987; Hansen et al., 1989; Shrader & Crothers, 1990).

Core histones could facilitate higher order folding through actions on neighboring DNA. The N- and C-terminal tails of the core histones are particularly likely candidates for having such a function: they are highly positively charged and are the sites of many posttranslational modifications (van Holde, 1989; Csordas, 1990). The N-terminal tails of all four core histones, and the C-terminal tails of H2A and H3, can be removed by trypsin and other proteases [see Bohm and Crane-Robinson (1984)]. Chromatin lacking the core histone N-terminal domains is not able to fold into the 30-nm filament state, and when devoid of histone H1 and the core histone N-terminal domains, it shows little or none of the ability to compact exhibited by chromatin that has been stripped of H1 but has intact core histones (Allan et al., 1982; see above). The stability of core particles against M^+-induced dissociation is not affected by the absence of any of the N-terminal tails (Whitlock & Stein, 1978; Ausio et al., 1989). Moreover, NMR experiments (Smith & Rill, 1989; Cary et al., 1978;

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* Author to whom correspondence should be addressed.

‡ Department of Chemistry.

§ Beckman Institute.

∥ Department of Biochemistry.

⊥ Address after Sept 1, 1991: Department of Biochemistry, Molecular Biology, and Cell Biology and Department of Chemistry, Northwestern University, Evanston, IL 60208-3500.

Department of Biophysics.

[1] Abbreviations: $[M^+]$, total concentration of all monovalent cations; PMSF, phenylmethanesulfonyl fluoride; BZA, benzamidine hydrochloride; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; WB, TE, PMP, various buffers (see Materials and Methods).

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Hilliard et al., 1986) have shown that the N-terminal tails of H2A, H2B, and H3 are highly mobile in the core particles. Taken together, these data indicate that the core histone tails are not involved in holding DNA onto the core particle as many workers had supposed. Chemical protection and protein–DNA cross-linking studies show that the N-terminal domain of H2B and the N-terminal and globular domains of H3 interact with linker DNA (Lambert & Thomas, 1986; Hill & Thomas, 1990; Bavykin et al., 1990). Thus, the core histones could contribute to higher order folding through effects on DNA, either by influencing the trajectory or mechanical properties of linker DNA or by interacting with DNA on neighboring nucleosomes.

Interactions between histones on neighboring nucleosomes (i.e., not involving DNA) could also be involved in chromatin folding. The existence of such interactions is manifested in several properties of histones and chromatin. Histone octamers, $H3_2$–$H4_2$ tetramers, and H2A–H2B dimers all exhibit a tendency to aggregate into higher multimeric assemblies [Klug et al., 1980; see Sperling and Wachtel (1981) and Royer and Scarlata (1989)]. When histone H1 is not present to prevent this, octamers tend to translocate along DNA in a process known as nucleosome sliding, leading to a product in which consecutive octamers are believed to be packed together face-to-face [for a review, see van Holde (1989)].

We have now extended our previous studies of linker DNA bending in dinucleosomes (Yao et al., 1990) to test the ability of linker DNA to bend in dinucleosomes that have been stripped of H1 (and of the H1 variant, H5). Dinucleosomes are studied because they allow the unambiguous detection of linker DNA bending through measurement of their nucleosome–nucleosome distance.

Materials and Methods

Materials. Chicken blood was obtained from Pel-Freeze; 123 bp ladders used as DNA size standards were obtained from Gibco/BRL. Yeast tRNA, micrococcal nuclease, and other chemicals were obtained from Sigma.

Preparations of Long Chromatin and Dinucleosomes. Long chromatin and dinucleosomes were prepared from chicken erythrocyte nuclei in WB (140 mM NaCl, 2 mM $MgCl_2$, and 15 mM cacodylate, pH 6.0) as described before (Widom, 1986; Yao et al., 1990).

Stripping of H1 and H5 from Long Chromatin, with Cation-Exchange Resin. The cation-exchange resin AG 50W-X2 was used to remove H1 and H5 from long chromatin, following the procedure of Graziano et al. (1988) precisely as described.

Stripping of H1 and H5 from Long Chromatin and from Short Oligomers, with High Salt Concentrations. NaCl (5 M) was added dropwise to a chromatin solution with stirring at 0 °C, to a final concentration of 0.66 M. For stripped long chromatin, the mixture was loaded on 5–30% sucrose gradients prepared in 0.66 M NaCl and centrifuged in an SW41 rotor at 41 000 rpm and 4 °C for 5 h. For stripped short oligomers, a solution of oligonucleosomes was treated with 0.66 M NaCl as described for long chromatin, and then centrifuged in an SW28 rotor at 28 000 rpm and 4 °C for 34 h. In a variant of this method, a second 5–30% sucrose gradient was run in TE buffer (10 mM Tris, pH 7.5, and 1 mM $Na_{2.5}$EDTA) to further purify stripped dinucleosomes.

Preparation of Stripped Dinucleosomes from Stripped Long Chromatin. Stripped long chromatin (at an A_{260} of 8) was digested with micrococcal nuclease (10 Worthington units/mL) at 37 °C for 8 min. The reaction was stopped by addition of EDTA to 2 mM. The digested mixture was then loaded on 5–30% sucrose gradients in TE and centrifuged in an SW28 rotor at 28 000 rpm and 4 °C for 36 h.

Stripping of H1 and H5 with tRNA. To remove H1 and H5 from the native dinucleosomes, yeast tRNA (100 mg/mL) was added at 30-fold mass excess to a dinucleosome solution in 40 mM NaCl and 3 mM EDTA, pH 8.0. The mixture was stirred at 0 °C for 48 h and then loaded on 5–30% sucrose gradients in TE buffer. The centrifugation was carried out in an SW28 rotor at 4 °C and 28 000 rpm for 29 h. Fractions containing stripped dinucleosomes were identified by 2% agarose gel electrophoresis and were then dialyzed against either 0.1× or 0.5× TE, or against PMP buffer (2 mM potassium phosphate, pH 7.5, and 0.1 mM NaEDTA).

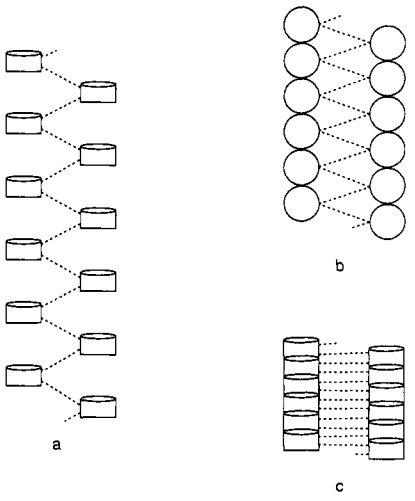
Dynamic Light Scattering. Scattering samples were prepared by mixing a stock solution of dinucleosomes at twice the desired final concentration, with an equal volume of buffer (0.1× or 0.5× TE) containing NaCl at twice the desired final concentration. The NaCl solutions were filtered through 0.2-μm filters prior to use, and samples were centrifuged to further remove dust immediately before measurement. Scattering data were collected as described before (Yao et al., 1990).

Electron Microscopy. Our procedure followed the alcian blue method of Sogo and Thoma (1989) as described previously (Yao et al., 1990). Samples were dialyzed into 1× or 2× PMP buffer, supplemented with additional NaCl as desired. Glutaraldehyde was added to 0.1% by dialysis, and fixation was allowed to proceed overnight at 4 °C. The samples were then dialyzed into 1× PMP without additional salts, adsorbed to alcian blue treated carbon films, washed extensively with deionized water, dried in ethanol, and shadowed with platinum. A negatively stained catalase crystal was used as a magnification standard.

Results

It is well-known that H1-stripped chromatin exhibits a Na^+-dependent compaction, albeit less so than for native chromatin at the same Na^+ concentration (Noll & Kornberg, 1977; Spadafora et al., 1979; Butler & Thomas, 1980; Fujiwara et al., 1989; Hansen et al., 1989). However, these results do not constitute evidence that this compaction arises from linker DNA bending, such as is known to occur for H1-containing chromatin (Yao et al., 1990). This point is illustrated in the hydrodynamic simulations of Figure 1. These simulations are for 12-mer oligonucleosomes, such as those studied by Hansen et al. Each nucleosome is represented as a disk, having a diameter of 11 nm and a thickness of 6 nm. The implied path or connectivity of linker DNA is illustrated by the dashed lines. Since there is no information available regarding the hydrodynamic properties of the histone "tails" or of linker DNA, these were omitted from the calculations. Model a illustrates the extended conformation believed to obtain in the starting dilute [Na^+]. Models b and c illustrate two ways in which the chain may be compacted to completely account for the extent of compaction measured by Hansen et al., yet in which the linker DNA has remained fully extended. The problem is that, while models b and c achieve compaction by packing nucleosomes together in space, the packed nucleosomes are not consecutive along the chain, and so the linker DNA need not be bent. We have previously shown that this ambiguity may be overcome by studying dinucleosomes (Yao et al., 1990).

Preparation and Characterization of H1-Stripped Dinucleosomes. Our experiments required that we prepare dinucleosomes from which the histones H1 and H5 have been removed. Several methods were tried to prepare these



MODEL	f/f_m	$D_{rel.}$
a	5.68	(1.00)
b	4.35	1.31
c	3.72	1.53

FIGURE 1: Hydrodynamic simulations for a chain of 12 nucleosomes (see text). Each disk was represented by 616 small spheres on a cubic lattice. The radius of the small spheres was chosen so as to make their aggregate volume equal to that of the disks. Frictional coefficients were calculated for a single (isolated) disk and for the 12-mers illustrated in the figure, using standard methods (Cantor & Schimmel, 1980). The dashed lines represent connectivities of the chains (i.e., implied DNA trajectories) but are not represented in the simulations. Model a represents an extended chain corresponding to the situation in dilute Na^+. In models b and c, there has been a significant compaction of the chain, but the linker DNA remains fully extended. Models a and c are shown viewed from the side and slightly above; model b is shown viewed from directly above. The results are presented as frictional ratios (f/f_m), in which the frictional coefficient for each 12-mer is divided by that for a single disk. Translational diffusion coefficients are proportional to $1/(f/f_m)$; relative values ($D_{rel.}$) are given here, with the value for model a set to 1.00.

"stripped dinucleosomes". We found that the method used to remove H1 and H5 is vital. Taking advantage of the facts that histone–DNA association is largely of electrostatic nature and that H1 and H5 are bound less tightly than core histones, H1 and H5 are usually selectively removed with negatively charged particles in appropriate salt concentrations or with elevated salt concentrations alone. Therefore, high salt concentrations, cation-exchange resin in low salt concentrations, DNA–cellulose, low pH, and tRNA have all been used to strip H1 and/or H5 (Ohlenbusch et al., 1967; Ilyin et al., 1971; Bolund & John, 1973; Oudet et al., 1975; Weischet, 1979; Libertini & Small, 1980; Allan et al., 1980).

Stripped dinucleosomes can be prepared from long chromatin by first stripping histones H1 and H5 and then carrying out further micrococcal nuclease digestion. We initially tried two procedures (and variants of them) to strip H1 and H5 from long chromatin, one using a cation-exchange resin and one using 0.66 M NaCl. When stripped dinucleosomes were prepared by further micrococcal nuclease digestion of such prestripped long chromatin, followed by sucrose gradient purification, they were found to be contaminated with naked DNA, and dinucleosomes were not well resolved, as judged by electron microscopy of samples fixed in 50–150 mM M^+ (data not shown). This observation is not due to an artifact of the electron microscopy. The method of sample preparation for electron microscopy that we used has been shown to protect against such artifacts for the particular chromatin concentrations and $[M^+]$ that we used (Sogo & Thoma, 1989; Thoma et al., 1979), and the identical procedure does not reveal naked DNA, and does reveal dinucleosomes, when used with stripped dinucleosomes prepared by a new method (see below). As an alternative method, we prepared native dinucleosomes first, then removed the H1 and H5 (without further use of micrococcal nuclease), and repurified the stripped dinucleosomes on sucrose gradients. In a variant of this method, native dinucleosomes were purified first, and salt was then added to strip H1 and H5, followed by another sucrose gradient to repurify the stripped dinucleosomes. All of these samples were found to be unsatisfactory for quantitation and further physical studies, because they were still contaminated by free DNA (data not shown).

tRNA has been used in previous studies to remove linker histone in rat liver chromatin (Ilyn et al., 1971; Noll & Kornberg, 1977; Spadafora et al., 1979). Our initial trail of this method using the established conditions, but on chicken erythrocyte chromatin, showed that the removel of H1 and H5 is incomplete; roughly one-fourth of the original amount of H1 and H5 remained (as judged from densitometer scans of Coomassie-stained polyacrylamide gels; data not shown), possibly due to the tighter binding of H5. Therefore, we tried adding more tRNA, to an excess of 30-fold in mass, but the removal of H1 and H5 was still incomplete (data not shown). We further modified the conditions to prolong the time of treatment with the increased amount of tRNA. Treatment of chromatin with a 30-fold excess of tRNA, in 40 mM NaCl and 3 mM EDTA (pH 8.0) at 4 °C for 48 h, resulted in the complete removal of H1 and H5, while retaining the core histones, as shown in the densitometer tracing of a protein gel (Figure 2a). The protein content is compared with long native chromatin (Figure 2b) to show that the contents of core histones are similar. (The possible slight change in the relative amount of histone H2A must be illusory rather than real, presumably due to the poor resolution of H3, H2B, and H2A, because analysis of the material extracted from the chromatin reveals the presence of H1 and H5 only, with no traces of core histones (data not shown).] Stripped dinucleosomes prepared by using this method look much better by electron microscopy (see below) than those prepared by the resin or salt methods.

The DNA contents of the stripped dinucleosomes was checked to ensure proper length and purity. Figure 2c shows the densitometer tracing of a 2% agarose gel of DNA obtained from stripped dinucleosomes. The DNA is free of monomers or other oligomers as judged by the low density at the corresponding positions. The mean length of DNA is 423 bp, obtained by careful measurement of the peak position of the sample and of the positions of the bands of the two lanes of length markers. This is the length that we expect for dinucleosomes prepared from chicken erythrocyte chromatin, which has a repeat length of 207–212 bp (van Holde, 1989). [Because the dinucleosomes are isolated from a population of mostly longer oligomers, they are not trimmed to the chromatosome positions (Noll & Kornberg, 1977; Simpson, 1978; Yao et al., 1990).]

Electron Microscopic Studies. Electron micrographs of stripped dinucleosomes prepared by using the modified tRNA method are shown in Figures 3 and 4. Provided that the stripped dinucleosomes are fixed for electron microscopy in high salt (Figures 3c and 4), the samples appear suitable for physical studies, and, in particular, they appear to be much better than those prepared by the other methods, in that free DNA is largely absent. Electron microscopy allows one to visualize the conformational change of dinucleosomes (Yao et al., 1990). We wanted to compare the state of compaction

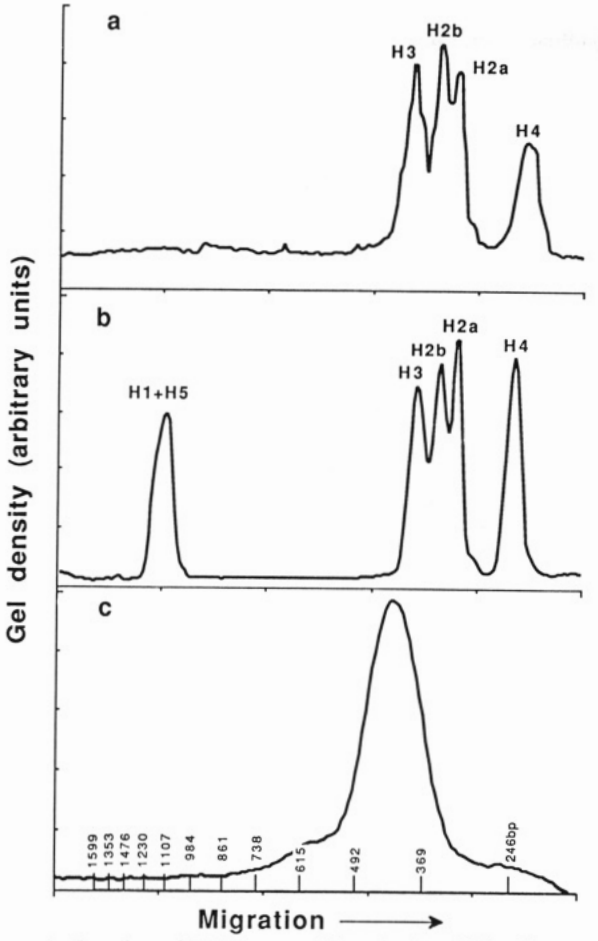


FIGURE 2: Protein and DNA composition of stripped dinucleosomes prepared by using the modified tRNA procedure (see text). Densitometer tracings of Coomassie blue stained 18% polyacrylamide gels of proteins present in stripped dinucleosomes (a) and in long native chromatin (b). (c) DNA present in stripped dinucleosomes. Densitometer tracing of a photograph of an ethidium-stained 2% agarose gel. Bands from the 123 bp ladder used as standards are marked as sticks.

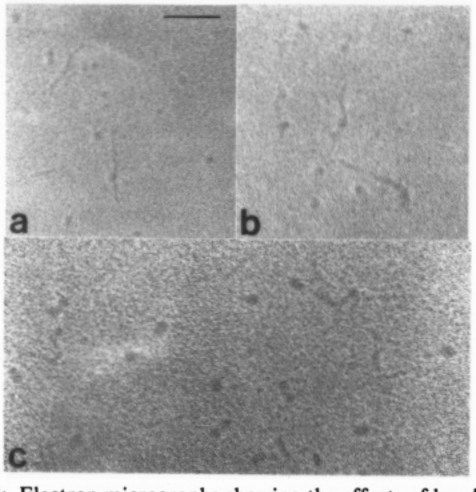


FIGURE 3: Electron micrographs showing the effects of low [M^+] on stripped dinucleosomes. Stripped dinucleosomes were fixed in (a) 1× PMP or (b) 2× PMP or (c) were first exposed to 0.1× TE and then brought back to 150 mM salt and fixed. Bar = 100 nm.

of stripped dinucleosomes in low and in high ionic strength conditions. However, images of molecules that were fixed in very dilute [M^+] look bad. Figure 3a shows images of stripped dinucleosomes that were fixed in 1× PMP; those in Figure 3b were fixed in 2× PMP. Nucleosomes are difficult to resolve,

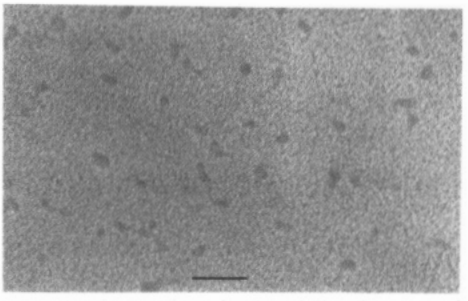


FIGURE 4: Electron micrographs of stripped dinucleosomes that were never exposed to very low [M^+]; fixed in 150 mM NaCl. Bar = 100 nm.

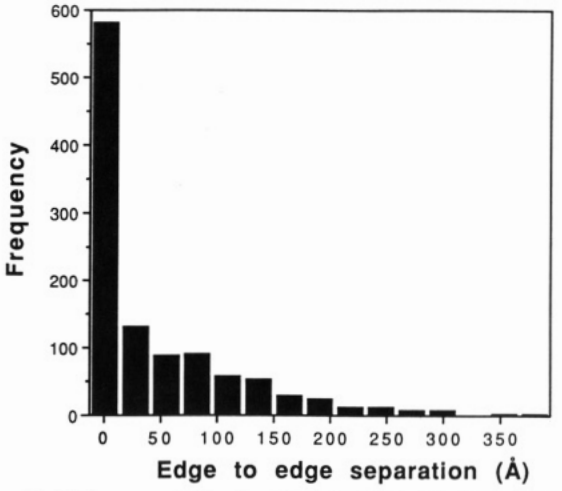


FIGURE 5: Histogram of edge-to-edge separation of the two nucleosomes in stripped dinucleosomes fixed in 150 mM NaCl. The measurements were made on prints at a total magnification of 179580×.

and both samples appear to contain naked DNA, which, for samples prepared by the modified tRNA method, was not present in the samples before preparation for electron microscopy in the very low salt (Figures 3c and 4). (The problem is a failure of the fixation procedure, which is known to happen at very low [M^+]; see below.)

Images of molecules that were exposed to dilute [M^+] but then returned to higher [M^+] before fixation for electron microscopy look much better. Figure 3c shows stripped dinucleosomes exposed to 0.1× TE and then brought back to 150 mM Na^+ before fixation. Images of stripped dinucleosomes that are kept in 5 mM M^+ (i.e., never exposed to the lowest [M^+]) and then fixed in 150 mM are shown in Figure 4. The images show that almost all of the stripped dinucleosomes are compact in these conditions. It is noteworthy that no free DNA was seen in any of the images from samples such as in Figure 4.

The width (long axis) of every image on prints from samples such as those in Figure 4 was measured. A total of 1096 images were measured, from several prints. The diameters of discernible individual nucleosomes within the dinucleosomes were also measured. The shadowing process enlarged the image of nucleosomes. Subtraction of the diameter of a shadowed nucleosome and the actual diameter of a nucleosome (11 nm; Richmond et al., 1984) from the measured width of the shadowed stripped dinucleosomes gives the edge-to-edge separation of the two nucleosomes in the dinucleosome. A histogram of the results is shown in Figure 5. Values of zero could be obtained if the two nucleosomes are in direct (edge-to-edge) contact, each lying with their faces on the grid. Some values are negative. Those dinucleosomes must have

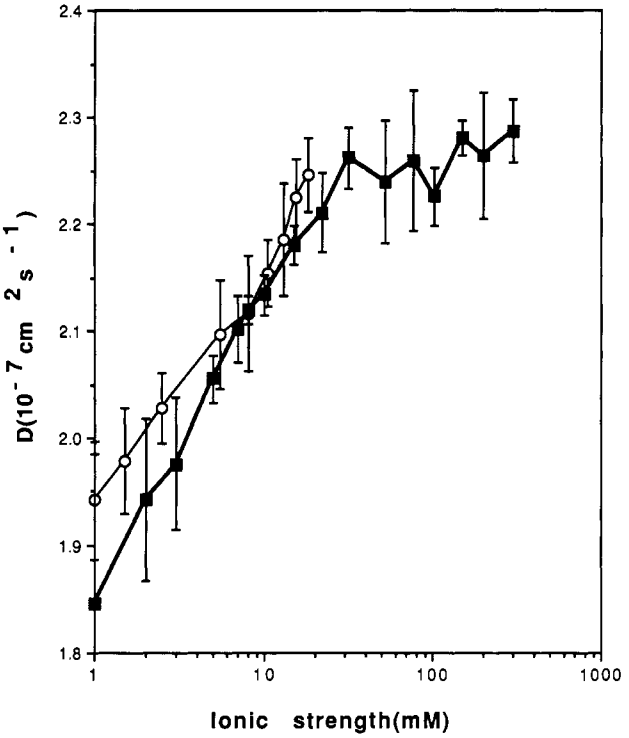


FIGURE 6: Translational diffusion coefficients of stripped dinucleosomes (■) as a function of ionic strength, measured by dynamic light scattering at 23 °C. Previous results for native dinucleosomes (Yao et al., 1990) are also shown (○) for comparison. Every symbol represents an average of four sets of measurements (four preparations). Vertical bars represent the standard deviation.

their nucleosome faces packed together, and could be lying on the electron microscope grid on their edges, or stacked like two coins. These values are included with the zero-separation data for the histogram shown in Figure 5. It can be seen that most stripped dinucleosomes are compact in high salt. If H1 and H5 were wholly responsible for the compaction of native dinucleosomes, the stripped dinucleosomes would have been extended, not compact (Yao et al., 1990), with a mode edge-to-edge separation equal to the linker length, which could be either 45 bp (150 Å) or 65 bp (220 Å) (see below).

Hydrodynamic Studies. In order to verify that the compact state of the stripped dinucleosomes detected by electron microscopy also represents their true solution behavior, we used dynamic light scattering to monitor the translational diffusion coefficients (D_t) throughout Na^+ titrations. Stripped dinucleosomes were dialyzed into two buffers. One contained 0.1× TE (pH 7.5) plus 4 mM NaCl. Its [M^+] is nominally "5 mM". Another contained 0.1× TE (pH 7.5) only, with an [M^+] of approximately "1 mM". For measurements with final [M^+] at or higher than 5 mM, samples starting in "5 mM" buffer were used. For those lower than 5 mM, samples starting in "1 mM" buffer were used. This distinction is to avoid dinucleosomes being unnecessarily exposed to very low [M^+] which might cause unfolding and dissociation (see above and Discussion). Figure 6 shows the diffusion coefficient of stripped dinucleosomes as a function of [M^+], compared with the data for native (H1- and H5-containing) dinucleosomes obtained previously (Yao et al., 1990). We were able to carry out the titrations up to 300 mM [M^+] without noticeable aggregation, in contrast to native dinucleosomes, which aggregate for [M^+] greater than ~25–30 mM. The diffusion coefficient increases monotonically as [M^+] increases. The trend is similar to that obtained previously for native dinucleosomes (Yao et al., 1990), but the transition is slightly shifted to higher ionic strengths.

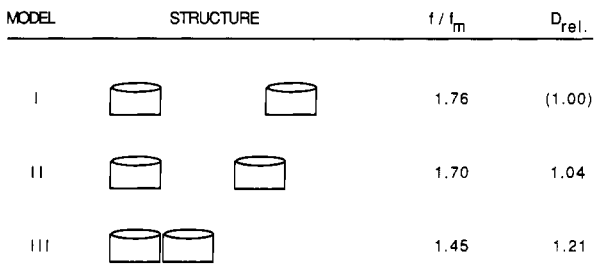


FIGURE 7: Hydrodynamic simulations. Each disk was represented by 616 small spheres on a cubic lattice. The radius of the small spheres was chosen so as to make their aggregate volume equal to that of the two disks. Frictional coefficients were calculated for a single (isolated) disk and for the pairs of disks illustrated in the figure, using standard methods (Cantor & Schimmel, 1980). Model I represents a stripped dinucleosome in which the 65 bp of DNA between the two (connected) core particles is fully extended. Model II represents an H1-containing dinucleosome; the separation between the two disks corresponds to the 45 bp linker being fully extended. Model III represents a case in which the two nucleosomes are in lateral contact. The results are presented as frictional ratios (f/f_m), in which the frictional coefficient for each pair of disks is divided by that for a single disk. Translational diffusion coefficients are proportional to $1/(f/f_m)$; relative values ($D_{rel.}$) are given here, with the value for model I set to 1.00.

At ionic strengths above 50 mM until 300 mM, the diffusion coefficient is unstable but hovers around 2.25×10^{-7} cm^2 s^{-1}. The diffusion coefficient increased about 22%, indicating a substantial degree of compaction.

Hydrodynamic Simulations. In order to approximately relate the observed magnitude of the increase in D_t for the dinucleosomes to an extent of compaction, we carried out computer simulations for a simple model (Figure 7). The dinucleosomes were represented as two disks, each having a diameter of 11 nm and a thickness of 6 nm, and the bending of linker DNA was simulated by varying the distance between them. Again, since thee is no information available regarding the hydrodynamic properties of the histone "tails" or of linker DNA, these were omitted from the calculations. The average linker DNA length for chicken erythrocyte chromatin is 45 bp, or 15 nm. A dinucleosome having a separation corresponding to 45 bp of linker DNA fully extended is represented in model II. We suppose that, when H1 and H5 are not present and in very dilute [M^+], an additional 10 bp of DNA at each end of the linker (which corresponds to the extra DNA at one end that relates a core particle to a chromatosome; Simpson, 1978) may also be extended; this is represented in model I. A compacted dinucleosome is represented in model III. Since we have no indication of a titration end point for the low [M^+] end, and because of the approximations described above, we cannot expect quantitative identity between the simulations and the experiments. Nevertheless, there is good agreement. For the lowest [M^+] investigated, our measured D_t's for the stripped dinucleosomes are ~5% lower than those for native dinucleosomes, as expected for model I compared to model II (4% increase). From the lowest to the highest [M^+] investigated, D_t for the stripped dinucleosomes increases by ~22%, as expected for model I compared to model III (21% increase).

Discussion

Preparation of Stripped Dinucleosomes. The quality of the preparations of stripped dinucleosomes was monitored by gel electrophoretic analysis of the proteins and DNA present, and by electron microscopy. Although samples prepared by further nuclease digestion of stripped long chromatin, or by high-salt stripping of dinucleosomes, look generally satisfactory in protein gels (data not shown), they look terrible by electron

microscopy, even in the high (i.e., moderate) salt conditions which produce the satisfactory images of Figures 3c and 4. Only samples prepared by stripping H1 and H5 with our modified tRNA method look satisfactory both in protein gels (Figure 3) and in electron micrographs (Figures 3c and 4). The quality of stripped chromatin prepared by using various methods of removing H1 (and its variants) has to be viewed carefully. These results have implications for previous and future studies. Stripped chromatin prepared by high-salt washing or by treating with cation-exchange resin may have significant quantities of free DNA, providing an undesirable substrate for physical studies. Possibly, stripping H1 and H5 from chicken erythrocyte chromatin is more problematic than stripping H1 alone from chromatin of other cell types because of the higher $[M^+]$ required for removing H5.

$[M^+]$-Dependent Compaction of Stripped Dinucleosomes. The translational diffusion coefficients measured by dynamic light scattering clearly show that, as $[M^+]$ is increased, stripped dinucleosomes become more and more compact. The measured compaction could be achieved, in principle, in any one of several possible ways. In the following paragraph, we rule out octamer sliding as a possible mechanism. Several explanations based on hydrodynamic artifacts, unfolding of nucleosomes induced by decreasing $[M^+]$, or further compaction of individual nucleosomes induced by increasing $[M^+]$ have previously been ruled out (Yao et al., 1990).

Histone octamers lacking H1 can slide together along a DNA molecule irreversibly, leading to an artifactual, closely packed structure [for a review, see van Holde (1989)]. This process generally requires long times at elevated temperatures and ionic strengths; it may be catalyzed or driven by the action of micrococcal nuclease. In the present study, stripped dinucleosomes were never exposed to greater than 40 mM NaCl during preparation. Micrococcal nuclease digestion was carried out prior to the stripping of H1 and H5. Furthermore, the stripped chromatin samples were always kept at either 0 or 4 °C. Therefore, conditions allowing sliding have not been approached in this study during the preparation of the stripped dinucleosomes. A separate question is whether sliding could occur when the dinucleosomes are adjusted to higher $[M^+]$ and/or 20–25 °C, for the dynamic light-scattering and electron microscopy experiments. Several lines of reasoning argue against this possibility: (1) The dynamic light-scattering measurement takes time, from a few minutes to about 2 h after initial exposure of the stripped dinucleosomes to elevated $[M^+]$. However, during this period, no systematic (or significant) changes in D_t were detected. (2) The observed compaction is complete by $[M^+] \sim 50$ mM (which is much lower than required for sliding to be detected at 20–25 °C, and still shows no time dependence to the D_t. (3) Sliding would lead to stretches of free DNA at one or both ends, and, if such DNA were present, it would be visible in the electron micrographs [data not shown (Results) and Figure 3a,b]; however, none is detected (Figure 4). (4) Finally, the sliding apart of H1-depleted octamers that have slid together has never been demonstrated; yet given the manner in which our stripped dinucleosomes are prepared, the compaction observed in this study must be reversible. We conclude that the observed increase in D_t and the compaction observed in electron micrographs are not due to sliding.

For these reasons, we conclude that the increase in D_t detected by dynamic light scattering in this study and the compact dinucleosomes observed in this study by electron microscopy are due to the bending of linker DNA, bringing the two nucleosomes of the stripped dinucleosomes into contact.

The path taken by the linker DNA is not known. If the two nucleosomes of a dinucleosome pack together as in the solenoid model for chromatin folding (Widom & Klug, 1985), then one possible trajectory would be for the linker DNA to bulge smoothly off the two nucleosomes in toward the solenoid axis.

Nucleosome core particles are known to unfold at low $[M^+]$ ($\sim$1 mM) (Burch & Martinson, 1980; Libertini & Small, 1980), near the low extreme of $[M^+]$ that we have employed. However, as discussed previously (Yao et al., 1990), our dynamic light-scattering studies do not reach the unfolding conditions found in those earlier studies. How are the diffusion measurements to be reconciled with the electron microscopic images of samples that were fixed in low $[M^+]$, which appear to show free DNA and structures that may be unfolded nucleosomes? The fixed samples are (necessarily) washed extensively in pure H_2O prior to shadowing. It is known that, for H1-stripped chromatin at low $[M^+]$, the fixation procedure fails to protect the structures against the unfolding which occurs at extremely low ionic strength ($\ll \sim 1$ mM) while for the same samples at higher $[M^+]$ the fixation procedure is adequate, possibly because of more extensive protein–protein cross-linking allowed by a more compact structure (Thoma et al., 1979).

Role of Histones H1 and H5 in Chromatin Folding. A surprising result from this study is that histones H1 and H5 are not essential for bending linker DNA, allowing two consecutive nucleosomes to pack together in space. Histones H1 and H5 do contribute to the free energy of compaction, as the M^+-induced transition is slightly shifted to higher $[M^+]$ in their absence. Since the titrations do not reach defined end points (and, additionally, have not been shown to be two-state), it is not possible for us to quantify the contribution of H1 and H5 to $\Delta G^\circ_{compaction}$; however, the data are consistent with an upper bound for this contribution of <1 kcal mol^{-1}. Electron microscopic and X-ray-scattering studies of long stripped chromatin show that the compact states which can be achieved lack the internal order characteristic of folded native chromatin (Finch & Klug, 1976; Thoma et al., 1979; Widom, 1989; Fujiwara et al., 1989). Thus, an important role of histones H1 and H5 is to select a single, ordered, compact conformation from a set of disordered compact conformations that are possible in the absence of H1 and H5. By contributing even only weakly to the free energy of compaction of the chromatin filament, H1 and H5 may accomplish this function.

Role of Core Histones in Chromatin Folding. While it was believed from previous work that attractive interactions exist between nucleosomes in long chromatin stripped of H1 and H5, it was not known which nucleosomes were interacting. One likely possibility was that linker DNA would not bend in the absence of H1 and H5 and that direct contact could occur only between nucleosomes that were distant (non-neighbors) along the chain.

We show here that stripped dinucleosomes are capable of a compaction that brings the two nucleosomes into contact. Long-range interactions are not possible in such molecules, since only two nucleosomes are present, separated by one linker. Thus, core histone octamers have an additional, previously unrecognized, property: they have the ability on their own (i.e., without H1 and H5) to bend linker DNA and bring consecutive nucleosomes into contact. Possible mechanisms exist enabling this function to be accomplished either by core histone octamer–DNA or by octamer–octamer interactions. Since long oligomers of stripped chromatin do not fold into ordered 30-nm filaments, it may be that the linker DNA bending or the nucleosome–nucleosome contacts which ac-

company linker DNA bending do not have a unique or sufficiently definite geometry, leading to disorder in the compaction of a longer oligomer.

It will be interesting to find out which core histone(s) or what part(s) of them are necessary for bending linker DNA, and to determine the trajectory of the linker DNA.

Relevance to the Folding of Long Chromatin. One key question that arises is whether studies of dinucleosomes are relevant to the folding of long chromatin. Four lines of evidence suggest that they are. First, as discussed previously (Yao et al., 1990), evidence exists showing that all four nucleosomes in tetranucleosomes may be simultaneously in contact, implying that the internal two linker segments must also be bent (Finch et al., 1975). Second, we have previously shown that the two nucleosomes of native dinucleosomes approach contact as $[M^+]$ approaches ~20 mM (Yao et al., 1990). The simplest interpretation of electron micrographs of long native chromatin taken throughout M^+ titrations is that consecutive nucleosomes are coming into contact as $[M^+]$ approaches ~15–25 mM (Thoma et al., 1979). Third, we show here that loss of H1 and H5 leads to a drop in D_t but that folding can still occur at elevated $[M^+]$. Long stripped chromatin behaves similarly (Finch & Klug, 1976; Noll & Kornberg, 1977; Thoma et al., 1979; Spadafora et al., 1979; Butler & Thomas, 1980; Hansen et al., 1989; Fujiwara et al., 1989), although, again, the nature of the folding observed for stripped long chromatin was not known. Finally, there exists evidence (Finch & Klug, 1976; Grau et al., 1982), confirmed and extended in our laboratory (J. Widom, P. T. Lowary, and J. Yao, unpublished results), that mononucleosomes and dinucleosomes can aggregate into fibers that closely resemble the 30-nm fibers of native long chromatin. Thus, it seems likely that the continuity of the DNA chain is not important for chromatin folding. For these reasons, we believe that studies of dinucleosomes are relevant to the structures and mechanisms of chromatin folding.

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REFERENCES

Allan, J., Hartman, P. G., Crane-Robinson, C., & Aviles, F. X. (1980) *Nature 288*, 675–679.

Allan, J., Harborne, N., Rau, D. C., & Gould, H. (1982) *J. Cell Biol. 93*, 285–297.

Ausio, J., Dong, F., & van Holde, K. E. (1989) *J. Mol. Biol. 206*, 451–463.

Bavykin, S. G., Usachenko, S. I., Zalensky, A. O., & Mirzabekov, A. D. (1990) *J. Mol. Biol. 212*, 495–511.

Bohm, L., & Crane-Robinson, C. (1984) *Biosci. Rep. 4*, 365–386.

Bolund, L. A., & Johns, E. W. (1973) *Eur. J. Biochem. 35*, 546–553.

Burch, J. B. E., & Martinson, H. J. (1980) *Nucleic Acids Res. 8(21)*, 4969–4987.

Butler, P. J. G., & Thomas, J. O. (1980) *J. Mol. Biol. 140*, 505–529.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Vol. 2, W. H. Freeman, New York.

Cary, P. D., Moss, T., & Bradbury, E. M. (1978) *Eur. J. Biochem. 89*, 475–482.

Csordas, A. (1990) *Biochem. J. 265*, 23–38.

Drew, H. R., & Calladine, C. R. (1987) *J. Mol. Biol. 195*, 143–173.

Dubochet, J., & Noll, M. (1978) *Science 202*, 280–286.

Felsenfeld, G., & McGhee, J. D. (1986) *Cell 44*, 375–377.

Finch, J. T., & Klug, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1897–1901.

Finch, J. T., Noll, M., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3320–3322.

Fujiwara, S., Inoko, Y., & Ueki, T. (1989) *J. Biochem. 106*, 119–125.

Grau, L. P., Azorin, F., & Subirana, J. A. (1982) *Chromosoma 87*, 437–445.

Graziano, V., Gerchman, S. E., & Ramakrishnan, V. (1988) *J. Mol. Biol. 203*, 997–1007.

Hansen, J. C., Ausio, J., Stanik, V. H., & van Holde, K. E. (1989) *Biochemistry 28*, 9129–9136.

Hill, C. S., & Thomas, J. O. (1990) *Eur. J. Biochem. 187*, 145–153.

Hilliard, P. R., Jr., Smith, R. M., & Rill, R. L. (1986) *J. Biol. Chem. 261(13)*, 5992–5998.

Ilyin, Y. V., Varshavsky, A. Y., Mickelsaar, U. N., & Georgiev, G. P. (1971) *Eur. J. Biochem. 22*, 235–241.

Klug, A., Rhodes, D., Smith, J., Finch, J. T., & Thomas, J. O. (1980) *Nature 287*, 509–516.

Lambert, S. F., & Thomas, J. O. (1986) *Eur. J. Biochem. 160*, 191–201.

Libertini, L. J., & Small, E. W. (1980) *Nucleic Acids Res. 8(16)*, 3517–3534.

Noll, M., & Kornberg, R. D. (1977) *J. Mol. Biol. 109*, 393–404.

Ohlenbusch, H. H., Olivera, B. M., Tuan, D., & Davidson, N. (1967) *J. Mol. Biol. 25*, 299–315.

Oudet, P., Gross-Bellard, M., & Chambon (1975) *Cell 4*, 281–300.

Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A (1984) *Nature 311*, 532–537.

Royer, C. A., Rusch, R. M., & Scarlata, S. F. (1989) *Biochemistry 28*, 6631–6637.

Shrader, T. E., & Crothers, D. M. (1990) *J. Mol. Biol. 216*, 69–84.

Simpson, R. T. (1978) *Biochemistry 17*, 5524–5531.

Smith, R. M., & Rill, R. L. (1989) *J. Biol. Chem. 264*, 10574–10581.

Sogo, J. M., & Thoma, F. (1989) *Methods Enzymol. 170*, 142–165.

Spadafora, C., Oudet, P., & Chambon, P. (1979) *Eur. J. Biochem. 100*, 225–235.

Sperling, R., & Wachtel, E. J. (1981) *Adv. Protein Chem. 34*, 1–60.

Thoma, F., Koller, T., & Klug, A. (1979) *J. Biol. Chem. 83*, 403–427.

Thomas, J. O., & Rees, C. (1983) *Eur. J. Biochem. 134*, 109–115.

van Holde, K. E. (1989) *Chromatin*, Springer-Verlag, New York.

Weischet, W. O. (1979) *Nucleic Acids Res. 7(2)*, 291–304.

Whitlock, J. P., & Stein, A. (1978) *J. Biol. Chem. 253*, 3857–3861.

Widom, J. (1986) *J. Mol. Biol. 190*, 411–424.

Widom, J. (1989) *Annu. Rev. Biophys. Chem. 18*, 365–395.

Widom, J., & Klug, A. (1985) *Cell 43*, 207–213.

Yao, J., Lowary, P. T., & Widom, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7603–7607.