Volume 5 Number 7 July 1978 Nucleic Acids Research

Alteration in nucleosome structure induced by thermal denaturation

V.L.Seligy and N.H.Poon

Molecular Genetics Group, Division of Biological Sciences, National Research Council, Ottawa, K1A 0R6 Canada

Received 19 May 1978

ABSTRACT

Mononucleosomes prepared from goose erythrocyte nuclei exhibited limited heterogeneity with respect to number of electrophoretic components, histones and DNA composition. The components differ slightly in ionic strength induced self-association. Thermal denaturation of each component gave only two dominant, highly cooperative, melting transitions, T" and T"'. Urea and trypsin were used to establish the differential lability of these two transitions. Comparison of the morphologies of the mononucleosomes at various stages throughout the melting profile indicated that the 13.3 ± 1.5 nm diameter mononucleosomes start to disrupt only in the latter half of transition T" and do not unfold until after reaching T"'. The resultant, open ended (17.4 ± 2.2 nm diameter) toroids are still largely negatively staining and much more uniform in shape if fixed simultaneously with gluteraldehyde.

INTRODUCTION

Electron microscopy and thermal denaturation are two of many techniques which have been used extensively to probe chromatin and its major substructural component, the nucleosome (1) or ν-body (2). The latter has been extensively characterized by physical and biochemical methods (see current reviews 3-6) and by electron microscopy using bright and dark field modes (1,2,6-15). A large number of attempts have been made to interpret the significance of the thermal melting transitions of chromatin and nucleosomes with respect to specific interactions of the DNA and protein moieties (16-35). Visualization of an altered nucleosome structure, such as found in the case for urea denatured nucleosomes (27,36), may be very useful for final interpretation.

In the present study we correlated visual changes in

nucleosomal morphology with the various thermal transitions of the DNA associated with nucleosomes. Controlled, thermally denatured mononucleosomes unfold to yield images similar to those described for urea denatured nucleosomes (27,36). The data are consistent with an arrangement of DNA similar to that recently predicted from neutron scattering and X-ray diffraction observations (3,6,15,37).

MATERIALS AND METHODS

Preparation of nucleosomes: Digests of intact goose erythrocytes (14) or chromatin (38) were carried out with micrococcal nuclease (E.C. 3.1.4.7, code NFCP, Worthington Biochemical Corp., actual assay 26.8 units per microgram) at 23°. The enzyme was assayed for histone proteolytic activity and found to be nil up to 24 hrs at 37°. Nuclear digests were terminated after about 15% acid solubility by addition of three volumes of ice cold 2 mM EDTA pH 8.0. The diluted digests, about 0.05 in ionic strength for nuclei, were gently stirred with a micromagnetic bar for 5 minutes at 0°C before enriching for chromatin fragments (range 3s < 50s) by centrifugation for 15 minutes at 2000 xg in a swinging bucket rotor. Chromatin fragments corresponding to bright field electron microscopically defined nucleosomes (7-11,14) were further isolated by ultracentrifugation (39) and ultrafiltration (14). The relative purity of the nucleoprotein preparations was assessed by comparison of electrophoretic mobilities with nucleoproteins from the original digest and DNA extracted from the pure mononucleosome fractions (14,40,41). Reference DNA markers were from λ phage and PM2 DNA digested with Hpa II and Hae III, respectively.

Proteins were analyzed on 12% and 20% polyacrylamide gels (42,43) using purified histones and cross-linked markers, 14,300 to 71,500 MW (BDH Biochemicals Ltd., Poole England).

Pure lyophilized histones consisting of H2A, H2B, H3 and H4 (1:1:1:1) all from mature goose erythrocytes (44) were weighed out, dissolved in glass distilled water and adjusted to pH 7.0 with addition of sodium phosphate to 5 mM. The protein stock was 2.5 mg/ml.

Thermal denaturation: Samples were analyzed by comparing the melting curves and first derivative plots obtained in 5 mM $NaPO_4$ pH 7.0. Thermal denaturation was done in a modified Gilford 240

equipped with a 6046 analog multiplexer and 2527 thermoprogrammer. The thermocuvette assembly was electronically heated. The method for digitalizing the data and computer analysis has been briefly described (24). For thermal denaturation studies involving electron microscopic analysis, the samples were heated in a manner equivalent to the 2527 programmer system. Specific experimental details are given in figure descriptions.

Electron microscopy: For routine electron microscopic work aliquots of unfixed or gluteraldehyde fixed nucleosomes, histones or DNA were placed on Kodak Photo-flo (0.1% v/v) prewashed or UV (254 nm) irradiated formvar coated grids (14) an equal volume of either uranyl acetate stain or 4% (v/v) glutaraldehyde. The sample grids were subsequently flushed with filtered distilled water, stained (in the case of fixed samples), air dried, and sandwiched between the formvar with a coating of evaporated carbon (45) at 5 x 10^{-4} mm Hg. The negative stain was usually 1% (w/v) uranyl acetate - 5% acetone; variations over the range of 0.005% to 5% aqueous uranyl acetate were examined. All images were recorded on Kodak Electron Image Plates using a Seimens Elmiskop 1A at 80KV. Optical enlargement of the original images was described previously using the Macrosystem and conventional enlarger (14). No chromatic abberations or image distortions were detected. The size of an object was determined from measurements either from accurately enlarged prints, using neutral contrast paper, or as we preferred, from the original negatives viewed by an optical microscope and an eye piece with a calibrated scale. The latter method is considered more precise because the original plates containing the primary electron images are immune to measurement loss in comparison to the enlarged prints which can result in as much as 10% deviation as the print contrast is increased.

Determination of temperature discrepancies between actual sample heating temperature and sample temperature at incipient transfer to electron microscope grids was facilitated by use of custom made microthermocouples which were geometrically fixed into five microlitre pipettes. The exact temperature and duration in seconds were recorded simultaneously using a Model 7001 AM Mosely Autograph X-Y recorder (Hewlett-Packard) equipped with

a Bio E 330 pen position adaptor; X-axis sweep was 0.25 seconds per cm; Y-axis temperature of expansion was 2.13°C per cm. For grid temperature measurements, microthermocouple probes were geometrically fixed at 0.5 mm above the center of each grid. This distance allowed for full contact with five microlitre or greater bubbles of fixative, stain or water placed on the grids prior to application of sample. The procedure was used to avoid direct contact with the probe and grid assembly. The grids, in turn, were rigidly held in position by their rims (≈0.5 mm from outer edge) with Dumont No. 5, super-fine, locking tweezers.

Results

Characterization of nucleosomes: Mononucleosomes used for thermal and electron microscopy studies are more limited in heterogeneity than recently reported (14,28,46-49). Densitometric scans gave only two major components (Fig. 1a) equivalent in mobility to about 194,700 to 247,500 daltons of DNA (calibrated with Hae III PM2-DNA restriction fragments F to N (Fig.1c) as assigned in ref. 50). A weight average molecular weight of 98,340 daltons was computed for the DNA (Fig. 1b). Each component contains histones H2A, H2B, H3 and H4. The dominant species, about 88% of the sample, corresponds to the "core particle" by definition of DNA base pair number (3,4,6). We earlier noted (14) that mononucleosomes, obtained by digestion of nuclei held intact by physiological saline buffer, were less heterogeneous than from nuclei or chromatin digested in low ionic strength buffer. Since the DNA digestion patterns are about the same (23,38,51) the heterogeneity we observed may be related to differences in self-association of nucleosomes induced by increased ionic strength (46). We examined this possibility using chromatin digested at low ionic strength and purified by sucrose gradient centrifugation using low and high ionic strength (14,39) buffer. Figure 2A shows the proportion of chromatin fragments or purified nucleosomes which do not aggregate at the various ionic strengths, relative to 150 mM NaCl nucleosomes. The solubility of the low ionic strength derived nucleosomes is similar to that observed by Olins *et al* (46) and Wittig and Wittig (32). Electrophoresis of the various precipitates and

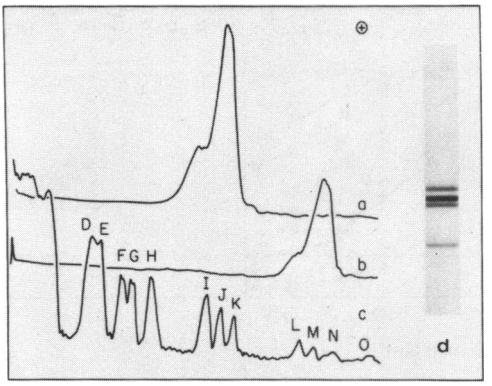

Figure 1. Characterization of mononucleosomes used in combined thermal denaturation and electron microscopy studies. Densitometric scans (580nm) of photographic negatives of ethidium bromide stained polyacrylamide slabs (0.3cm x 20cm) were made: (a) mononucleosomes (125 μg DNA), (b) DNA from nomonucleosomes (50 μg) and (c) Hae III restriction fragments of PM2 DNA (15 μg, 3X chart expansion). The 4% (w/v) polyacrylamide gel slabs were electrophoresed with continuous buffer circulation. Calibration of restriction fragments was based upon pre-designated sizes (50). (d) disc polyacrylamide gel (0.5 cm x 10 cm) showing amido black staining proteins (Top to bottom, H3, H2B, H2A and H4) from main nucleosome band; the minor component also contains the same histones.

soluble fractions, shown in Fig. 2B, indicates as ionic strength increases there is a preferential selection of mononucleosomes over oligomeric forms and a reduction in hererogeneity of mononucleosomes. To further qualify the significance of the individual mononucleosome components with respect to protein, DNA size and thermal melting behaviour, nucleosomes were preparatively electrophoresed and individual components eluted and examined. Figure 3A shows that the nucleoprotein from the various bands retain their relative mobility, which is approximately related to the computed DNA base pair number. The variations are consistent with other reports (14,28,46,49). The content of nonhistone is very low (<2%). This has been already noted (14). As shown in Figure 3B, the major variation in protein content centers on whether H1 and/or H5 are present or not; the 125 base pair fragment may have more H3 and H4 associated with it. Differential solubility of the mononucleosome population is apparently related to H1 and H5 (Fig. 2) and some nonhistone proteins (54).

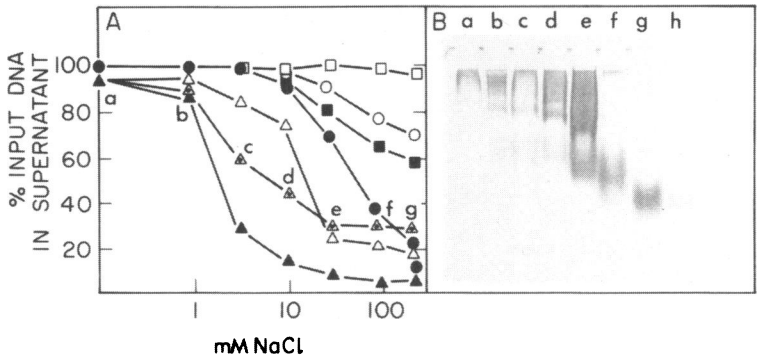

Figure 2. Effect of ionic strength on self-association or aggregation of nucleosomes. A, low gravity (2000 x g, 20 minutes) separation of aggregated nucleoprotein as a function of salt concentration. (▲) chromatin digested with micrococcal nuclease (4×10^{-4} units/µg DNA) for 5 minutes at 23° (0.4% acid soluble (38), 8± 3% insoluble chromatin in 1 mM EDTA pH 8.0 at 2000 x g 20 minutes). Chromatin digested to 15 - 18% acid solubility in low (38, △) and high (14, ▲) ionic strength buffer. Nucleosomes associated with (●) and without (○) histones H1 and H5 (see Fig. 3, c and d). Nucleosomes from 11.5 S monomer peak from chromatin digested at low ionic strength (see △) and fractionated by 5-20% sucrose gradients in 1 mM EDTA pH 8.0 (■) or 100 mM NaCl-50 mM Tris-HCl-1 mM EDTA, pH 8.0 (□). B. Electrophoretic analysis of DNA prepared from chromatin aggregates collected in experiment, ▲; a to g refer to precipitates in panel A; h is the 150 mM NaCl soluble mononucleosome. Photograph is a reverse print of a 3.5% polyacrylamide slab gel.

Melting profiles: The derivative melting profiles of mononucleosomes treated or untreated with urea and/or trypsin are shown in Figure 4. These data show that the melting profile of isolated mononucleosomes is composed of two components, referred to here as T" and T"' . This agrees with recent reports (22,23,27-35). Urea treatment, which has been shown to disrupt chromatin and nucleosomes (16,23,27), preferentially eliminates T"' over T"; above 6M urea a DNA-like component (T°) occurs (Fig. 4), which represents about 8 to 12% of the total hyperchromicity. In our experiments urea neither caused a change in total hyperchromicity of the components nor any noticeable increase in turbidity (27). Treatment of mononucleosomes with trypsin resulted in the preferential elimination of T"' over T". This is also shown in Figure 4. The extent of elimination of T" could be enhanced if urea was included; urea added before or during proteolytic digestion was very effective in this elimination. Trypsin and urea

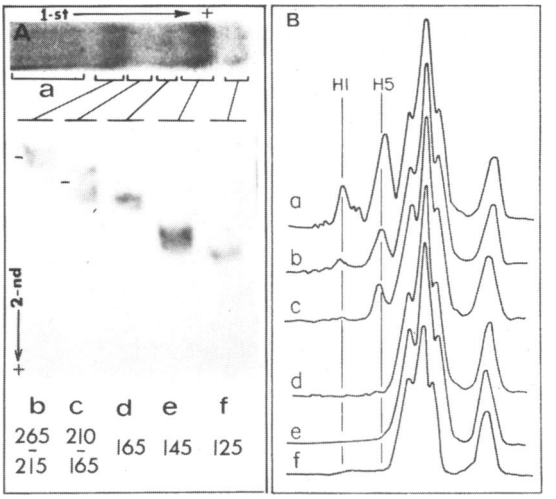

Figure 3. Subfractionation and protein characterization of mononucleosomes. A., preparative electrophoresis of a low speed fraction of micrococcal nuclease digested chromatin (0.05 ionic strength soluble fraction in Fig. 2 (▲) and ref. 14, Fig. 1a). The 3.5% (1 cm x 20 cm) polyacrylamide slab gel contained 2.5 to 3.0 mg of sample per 3 cm slot. Electrophoresis was for 14 hours at 30 mA. Ethidium bromide fluorescent bands were exercised and contents electrophoretically eluted. The lower slab (0.3 cm x 20 cm) shows the purity of the components. The base pair assignments of the DNA from each component were from calibrated fragments (Fig. 1c). B, densitometric scans (575 nm) of histones from each component. Electrophoresis was at pH 2.7 (43,44).

treatment generated two main types of DNA melting: one with melting properties equivalent to deproteinized DNA and a second, broad melting component of value $\leq T"$. The latter can only be completely removed by addition of sodium dodecylsulphate and further DNA purification (Fig. 4). In all experiments, using the same nucleosomal starting material and appropriate blank controls, the difference in total hyperchromicity between native, urea, and urea-trypsin treatments was less than 6.8%. Trypsin treatment alone was about 8.0%. The sharp co-operative character and near equivalent hyperchromic values suggest that no major single strand stacking has taken place, except possibly for trypsin treated material.

Figure 5 shows the results of a study using the electrophoretically fractionated mononucleosomes of Figure 3 and oligomers from the ionic strength precipitation experiments (Fig. 2). Firstly, it is apparent from the thermal derivative plots that

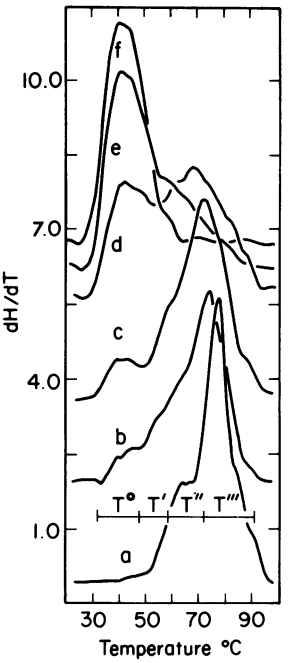

Figure 4. Derivative thermal denaturation profiles of mononucleosomes before and after treatment with urea and/or trypsin. The plots (24) were obtained with 1.5 x 10^{-4} M DNA-P in 5 mM PO_4 at pH 7.0. Mononucleosomes are from experiment in Fig. 1. a, untreated; b, urea 6 M for two hours and c, 8 M; d, trypsin 0.003 w/w at 23° for 1 hour at pH 7.0 and e trypsin with 6 M urea added during digestion, and f, DNA from nucleosomes in 6 M urea. Experiments were in duplicate; details are in Methods.

melting of nucleosome fragments are similar. Secondly, as DNA fragment size is increased there is a slight shift of the T'" transition and an appearance of minor low (<T") and high (>T"') melting transitions. Thirdly, component T" is slightly augmented and less co-operative (measured by half peak height) in submonomer particles (the 125 base pair unit); it becomes increasingly contiguous with T"' as DNA fragment size increases. Since some fragments have either H1 or H5 associated with them, the shift of T" and T"' may be due to these proteins. However, T" broadens and both T" and T"' shift to lower values when 1 mM PO_4 buffer is used.

Controls for correlating visual and thermal studies: As major controls we previously studied the patterns and images found in the absence of sample, stability of sample to uranyl salt concentration and contamination due to free histone (14). The drying pattern with buffer alone prepared and stained in the

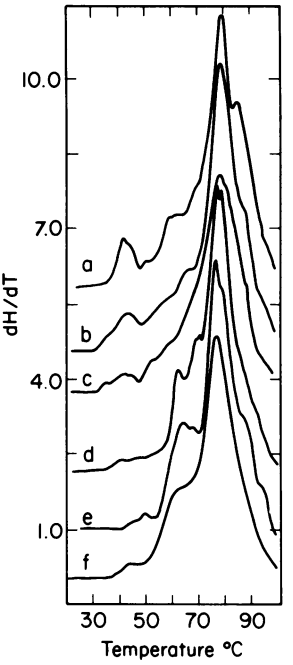

Figure 5. Derivative thermal denaturation profiles of mononucleosomes, dinucleosomes and oligomeric fragments. Plots a and b correspond to fragments obtained from experiments in Fig. 2 indicated by symbols ▲ and △; c to f plots correspond to b, c, d, and f components in Fig. 3.

same way as for samples, is neither random nor the same as when sample is included. The deposition pattern is altered as the concentration of sample per grid is increased (14). At higher magnifications no images comparable to the ones for nucleosomes could be detected with buffer alone at any temperature. This possibility was extensively studied because transfer of heated sample from thermal cuvette to individual electron microscope grids could introduce artifacts due to heat fluctuations of sample and grid. The variables associated with sample transfer were qualified for each increase in temperature by use of microthermocouples inserted appropriately in the capillaries of the temperature equilibrated micropipettes and onto the grid surfaces. A detail of this description is presented in the Methods and in the legend of Figure 6. From Figure 6A and C it can be seen that the average time required for sample transfer and treatment on the grid is very short, 1.63 ± 0.3 seconds and 2.13 ± 0.2 seconds, respectively. Loss of heat during transfer is charac-

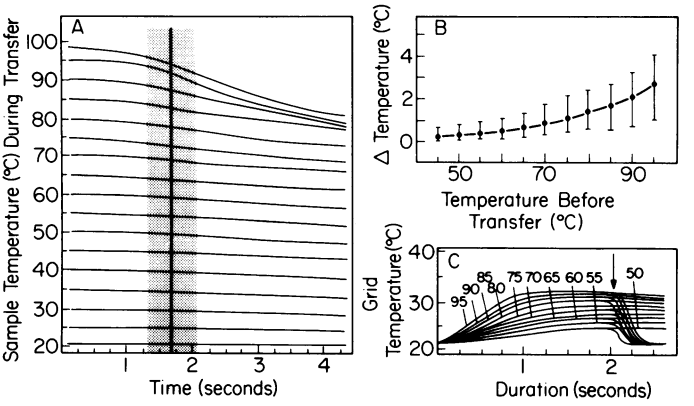

Figure 6. Determination of temperature differentials during specimen heating and transfer to electron microscope grids. A. Change in sample temperature during transfer from thermal cuvette to individual grids. Vertical shaded bar indicates all points where samples were released from 5 microlitre pipettes equipped with micro-thermocouple probes in the capillaries. B. Discrepency in sample temperature before transfer and incipient release of sample onto electron microscope grids. Data was obtained from graphs in A using an averaged transfer time of 1.63 ± 0.30 sec, where the number of repetitions was 85. C. Resultant increased grid temperatures following application of samples at various sample transfer temperatures shown in A and B. Vertical temperature drops indicate points in time and final grid temperatures when washing of grids with staining solutions or water occurred. Maximum grid temperature, which occurs at 95°C, was 32.4 ± 0.8°C.

teristic for each starting temperature; it does not become significant until above 70°C (Fig. 6A and B). At 97°C the average heat loss during transfer was 2.8 ± 1.6°C. Similarly it was found that sample transfer temperature below 50°C did not significantly alter the original grid temperature; maximum grid temperature at 97°C was 8.4 ± 0.8°C above ambient room temperature. Even at this temperature the exposure was less than 1.5 seconds after which the grids were flushed with buffer (Fig. 6C). By using the data in Figure 6 and judiciously comparing grids without sample, before and after heating, we concluded that elevated temperatures alone cannot generate morphological staining artifacts.

Cooling of sample during transfer could result in possible renaturation of some denatured material. To determine to what extent this might occur chromicity changes at 260 nm were monitored as nucleosome samples were program heated to various

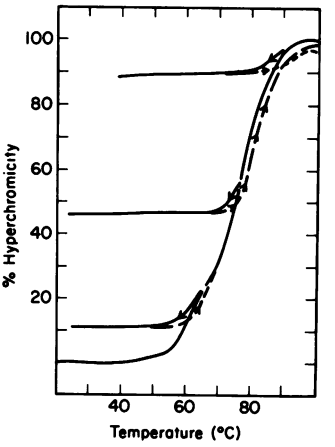

Figure 7. Analysis of nucleosomal-DNA denaturation and renaturation. Samples of nucleosomes (1.5×10^{-4} M DNA-P), in triplicate, were melted to various extents (a, b and c), cooled rapidly to ambient temperature (23°) and re-heated by way of a thermal program to 97°. Hyperchromicity of control sample, denatured in one continuous phase was 33.7% (——). Symbols (←) and (→) indicate cooling and re-heating phases, respectively.

temperatures, cooled to room temperature and program re-heated to 97° (melting of all DNA). The results, presented in Figure 7, are only for the beginnings and ends of the T" and T"' transitions. Each of the transitions has some potential for hypochromicity (after correcting for water expansion); the beginning of the T" transition is most affected and T"' the least. Hyperchromicity due to re-heating the nucleosomal material is almost totally recoverable for the former, but not for the latter. Therefore combining the results in Figure 6A, B and C with those of Figure 7, it is likely that only the material at the beginning of transition T" might be affected by transfer of sample during heating. However, at these temperatures sample heat loss, prior to fixation on the grids, is very small.

To determine to what extent the morphological and spectroscopic changes, which occur during nucleosomal-DNA denaturation, may be attributable to free histone-histone interactions alone, pure, nucleic acid free, histones were heat treated and simultaneously monitored for morphology. The results of these experiments can be briefly summarized as follows: 1) a cooperative heat induced hyperchromic change can be clearly detected at 275 nm; 2) the major transition occurs about 10°C

Table 1. Statistical measurements of native and denatured Mononucleosomes

Form	N	O.D. (nm ± s.d.)	I.D. (nm ± s.d.)	Contour Length[a] (nm ± s.d.)	Percent B-Form[b] Length
Native	550	13.3 ± 1.5	1.68 ± 0.8	-	-
Denatured	405	17.4 ± 2.2	9.8 ± 2.0	42.9 ± 6.3	86 ± 4.8

[a]Computed by direct length measurement of linear forms or by formula, $\pi((O.D. - I.D.)/2 + I.D.)$.

[b]Computed from weight average molecular weight 98,340 d ± 3%.

above that of purified DNA from nucleosomes; and 3) the morphology of the histones, which have been described (14), change, but the images do not resemble those obtained with nucleosomes at any of the elevated temperatures.

Image and staining characteristics of native and denatured nucleosomes: Figure 8 shows the general negative staining properties of mononucleosomes at 23°C, prior to thermal denaturation. All images were particulate, either circular or prolate elipsoids. A size analysis of the inside and outside diameters is summarized in Table I. The dimensions of the particles are in good agreement with our earlier detailed study (14) and those obtained from chick erythrocytes (1,2,11). The bulk of the nucleosomal images changed very little in shape or stainability as the temperature is raised from ambient (23°C) to 70°C. This holds whether the sample is fixed in gluteraldehyde immediately prior to staining or directly stained without fixation (Fig. 8b,b'). A significant amount of swelling and tendency of the nucleosomes to aggregate was noticed at the latter part of transition T" (Fig. 8b,b'). However, no further change was noted until T"' was reached; at this point, the average nucleosomal morphology has drastically altered and both inner and outer diameters have increased (Fig. 8c,c', Table I). The average fiber length from perimeter measurements is less than the length computed from the weight averaged base pair number assuming B-form duplex DNA (Fig. 1) (1,27).

A detailed comparison of the unfixed and fixed material indicates that the unfixed material yields more linear and U-

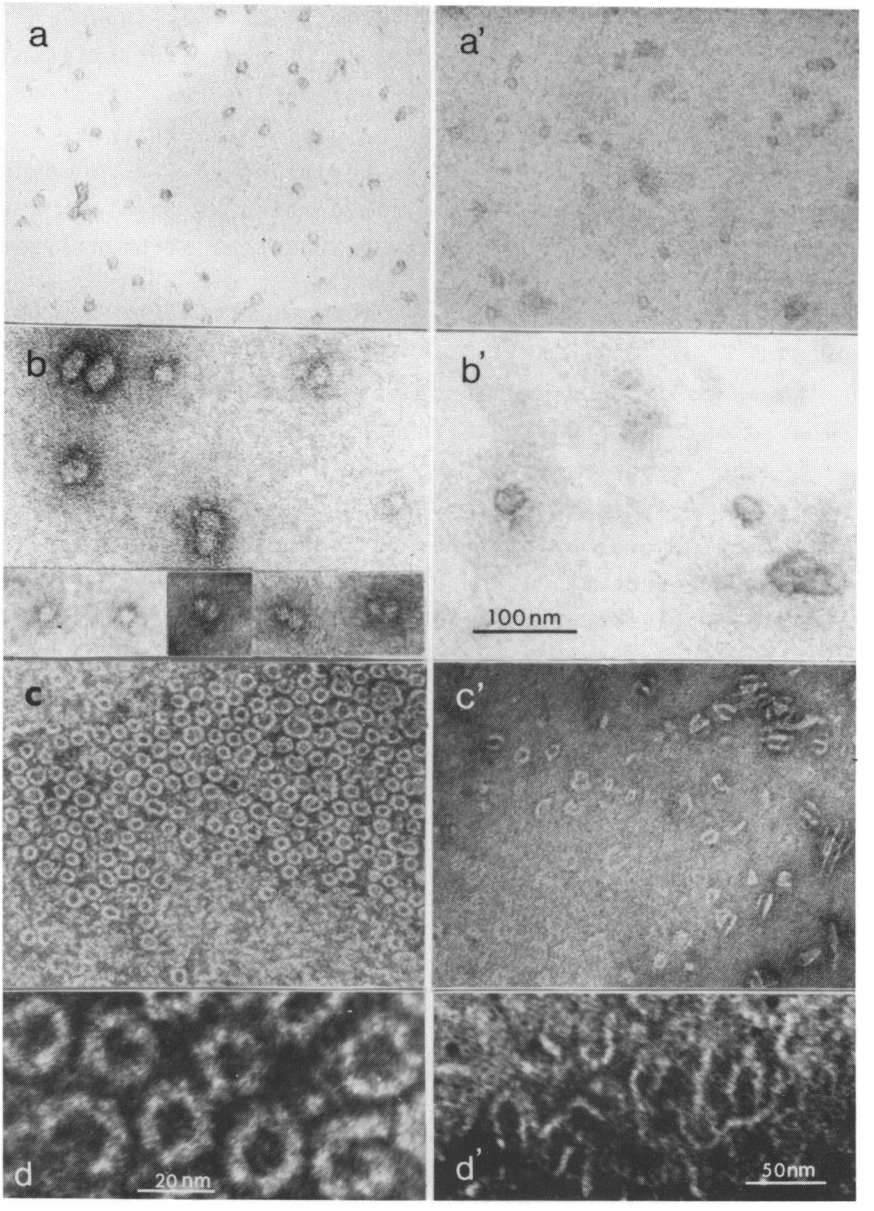

Figure 8. Morphological changes in mononucleosomes at various stages of heat denaturation. Duplicate samples removed from thermal-regulated cuvette at 2°C intervals and transferred to grids. Procedures are described in methods and in Fig. 6. a, a^1, 23°; b, b^1 and c, c^1 correspond to end of T" and T"' transitions shown in Fig. 4., d, d' are high magnifications of c, c'. Gluteraldehyde was omitted in a', b', c' and d'.

shaped structures than open ended circles. This is shown in the high magnifications of Figure 8d,d'. Close scrutinization of grids with samples from each temperature interval indicated that a minor component with similar morphological features seen in Figure 8b to c also occurs at the beginning of T". However, these ringed structures may have disintegrated as the temperature was increased, because they could not be detected at higher temperatures.

DISCUSSION

The composition of mononucleosomes used here specifically for combined melting and visualization studies is in good agreement with other reports (3-6). We have shown that mononucleosome variation (14,27) can arise through differences in self-association of monomer or oligomer nucleosomes by increased ionic strength and presence of H1 and H5 associated proteins. The heterogeneity established by electrophoretically subfractionated mononucleosomes is in agreement with those from chicken (27). The extremely low nonhistone protein content of the monomers is not unusual because: 1) mature chicken or goose erythrocyte nuclei contain very low amounts of nonhistone in comparison to nuclei from immature cells or other tissues (52,53) (especially in classes below 68,000 daltons) and 2) the bulk of the high molecular weight residual proteins, which appear to be mostly associated with nuclear membranes or matrix, are relatively insoluble unless extensively digested with micrococcal nuclease (54). Since the initial preparation of this manuscript several related reports have been published which further document the differential self-association of monomers and oligomers (32,55-37). These studies provide a basis for interpreting the earlier observed differential sensitivity of erythrocyte chromatin (heterochromatic and euchromatic structure) to both mono and divalent cations (57-60) before and after sequential removal of proteins (52,53,59).

The melting profiles of electrophoretically defined mononucleosomes and select oligomeric fragments obtained by ionic strength precipitation methods clearly indicate that only two major transitions (T" and T"') are associated with mononucleosomes. As shown here and in part elsewhere (22,23,27,28,30-34),

thermal transitions $\leq$ T" and>T"' are peculiar to nucleoproteins of large DNA length ($\geq$ 265 base pairs) and increased protein complexity. However they are not necessarily due to previous degradation (21, 25,28,30,32). Allowing for solvent differences, our results are very similar to those obtained by Mandel and Fasman (22), Lewis (28), Wittig and Wittig (32). They are also in excellent agreement with the highly complementary physical studies by Weichet and co-workers (68) that appeared on submission date of our original manuscript. The combined studies clearly indicate that the presence of transitions T" and T"' cannot be directly accounted for by variations in DNA length, presence or absence of lysine-rich histones (H1, H5) or nonhistone proteins; but, co-operativity and resolution of these transitions particularly T", can be influenced by the preceding parameters as well as by differences in ionic strength of the melting buffer. We routinely used 5 mM $NaPO_4$ at pH 7.0, because much less visual unfolding of the mononucleosomes (14) takes place than at lower ionic strengths such as with 0.1 mM EDTA,and T" and T"' are still distinct. Weichet *et al* (68) have also recently observed the latter effect using sodium cacodylate buffer. The thermal reversibility of T" and differential sensitivity of T"' with urea and trypsin treatment of nucleosomes will be discussed further in reference to nucleosome unfolding.

Olins and co-workers (27) have skillfully shown by electron microscopy that urea induces unfolding of nucleosomes. A similar claim has been made by Woodcock and Frado (36). Simple storage of nucleosomes in low ionic strength buffer can also promote denaturation (14,66); the nucleosomes unfold into two major halves in the absence of any major DNA or protein degradation. In this study we show for the first time that during thermal denaturation purified mononucleosomes undergo reproducible morphological changes which are generally nonreversible. First, the morphological changes occur whether or not the nucleosomes are fixed in gluteraldehyde prior to uranyl acetate staining. Second, the major change is from the typical compact toroid shape (Fig. 8, Table I and ref. 2,7,10, 11-15) to the large circular, U-shaped, crescent-shaped, or extended structure of length about 90% of a weight average native DNA B-form equivalent. Third, the major portion of these morphological changes is seen only after reaching transition T"'. Unlike formal-

dehyde (67), which was purposely avoided here, we do not feel the highly regular ring structures obtained with gluteraldehyde fixation are artifacts; basically the same images were obtained by two independent methods of staining. However, the presence of linear, extended forms in unfixed samples supports our initial contention that fixation would help stabilize the delicate denatured nucleosome structures from mechanical disruption during staining, washing and drying of grids. The inherent rigidity of small DNA segments of this order (50), particularly in the denatured state, would tend to generate linear structures such as already seen for low ionic strength (14,66) and urea (27,36) denatured nucleosomes. A detailed study of the unfolded structures at high resolution, and an analysis of the periodic, negatively staining elements, which are seen along the open ringed images, is being carried out using image reconstruction methods. The ringed structures are highly compatible with recent X-ray diffraction data (15) and corresponding subunit models (37,64).

Several studies (21,22,26-30,61,68, this study) indicate that the major melting component of chromatin is likely related to the principal transition (T"') of the simple mononucleosome. However, the shear complexity of DNA-helix stability in chromatin prohibits definitive assignment of other melting transitions at this time. Nevertheless, an earlier model, derived from chromatin work (18), has considerable appeal in that the two major transitions are postulated to originate from the disruption of protein-protein interactions and melting of protein-free DNA (Tm^1) and from the destruction of the supercoil and DNA strand separation (Tm^2). Our visual and hyperchromic studies and the recently reported hyperchromic, circular dichroic and calorimetric studies on chicken erythrocyte nucleosomes by Weichet et al (68) provide new insight into the thermal denaturation process of chromatin subunits. Overall, the bi-phasic melting of core particles is interpreted as a denaturation of about 40 base pairs in the first phase, followed by a massive unfolding of the native structure of a tight histone-DNA complex, which frees the remaining 100 base pairs in stacking (the second phase). Our study clearly shows that nucleosomes begin to alter original toroid shape only during the latter third of T" and do not actually co-operatively unfold until T"' is reached. The unfolding pattern is highly consistent

with the predicted 1 3/4 loop or supercoil arrangement of nucleosomal DNA (15,37). From the circular dichroic data (68) it is further apparent that no change in secondary structure of the protein core takes place at T". Since the core is stable, it therefore may serve to lock the major part of the DNA into a rigid complex thus allowing only certain regions to gain limited mobility at $\leq$ T". This would explain the reversibility of T" that we both observe, albeit in our case complete hypochromicity occurred only in the first third of T". It is important to stress that the first stable morphologically altered state of the nucleosome occurs in the latter third of T". It is there that the toroids become measureably expanded, frequently aggregated into clusters of 2 to 4 and ends of loops are occasionally detectable. Since secondary structure of histones is located in the more hydrophobic domains, which are the regions that interact with each other (3-6,28,31,61, 63,65,68), the aggregation property may be a direct measurement of the alteration in histone structure that takes place at this stage (68).

Unfolding of nucleosome secondary structure by urea has been earlier shown by electron microscopy (27,35,60), hydrodynamic and circular dichroic studies (23,35,60). Physical changes in mononucleosomes following trypsin treatment have also recently been reported (31). The data are explained on the basis of a structural unfolding of nucleosomal DNA whereby part of the DNA retains the properties within intact core particles and the remaining portion behaves much like free DNA. We note that the melting data of Olins and co-workers (27) and Lilley and Tachell (31) exhibit a similar preferential loss of transition T"' over T" that we observed here. However, no visual studies on the unfolding of trypsin treated nucleosomes is currently available for more rigorous interpretation. From present work, which correlates unfolding of the supercoiled DNA with T"', preferential loss of T"' because of urea, trypsin or urea and trypsin treatment of nucleosomes would be expected. It is tempting to conclude that the presence of T"' can be used as a direct indicator, at all times, for morphologically intact nucleosomes. However, our recent shearing studies (in preparation) indicate that this is not always true, particularly if no T" is present. Similar caution should be applied when inter-

preting the origin of transitions $\leq$ T". In addition to T" arising from the melting of a limited number of base pairs within the nucleosome (68), it could also arise from the melt-out of a minor nucleosome or nucleosome-like population which lacks thermal stability in the denatured state as exhibited by denatured core nucleosomes at temperatures > T"'. Close scrutinization of micrographs obtained from the early portion of T" of a number of samples indicates that this is possible. Therefore, for some nucleosome elements there may be a difference in either the types of protein (species or charge modifications) or the way core proteins are associated with DNA sister strands (intra or inter-strand cross-linking) after denaturation. A study of single strand availability and DNA sequence polarity before, during and after thermal denaturation of native nucleosomes may be useful approaches for investigating this possibility.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. C.V. Lusena (N.R.C.C.) and A.R. Morgan (University of Alberta) for providing us with λ and PM2 DNA. We are extremely indebted to the expert technical support skills of M.J. Dove, L. Sowden and R. Whitehead. This is NRCC contribution No. 16572.

REFERENCES

1 Olins, D.E. and Olins, A.L. (1974) Science 184, 330-332
2 Oudet, P., Gross-Belland, M. and Chambon, P. (1975) Cell 4, 281-299
3 Kornberg, R.D. (1977) Ann. Rev. Biochemistry 46, 931-954
4 Felsenfeld, G. (1978) Nature 271, 115-122
5 Li, H.J. (1975) Nucleic Acids Res. 2, 1275-1289
6 van Holde, K.E. and Isenberg, I. (1975) Acc. Chem. Res. 8, 327
7 van Holde, K.E., Sahasrabuddhe, C.G. and Shaw, B.R., van Bruggen, E.F.J. and Arnberg, A.C. (1974) Biochem. Biophys. Res. Commun. 60, 1365-1370
8 Olins, A.L., Carlson, D. and Olins, D.E. (1975) J. Cell Biol. 64, 528-537
9 Bakayev, V.V., Melnickov, A.A., Osicka, V.D. and Varshavsky, A.J. (1975) Nucleic Acids Res. 2, 1401-1419
10 Finch, J.T., Noll, M. and Kornberg, R.D. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 3320-3322
11 Woodcock, C.F.L., Safer, J.P. and Stanchfield, J.E. (1976) Expt'l Cell Res. 97, 101-110
12 Langmore, J.P. and Wooley, J.C. (1975) Proc. Nat. Acad. Sci. U.S.A. 72, 2691-2695
13 Poon, N.H. and Seligy, V.L. (1977) Proc. of Microscopical Society of Canada 4, 56-57

14 Poon, N.H. and Seligy, V.L. (1978) Expt'l Cell Res. 113, 95-110.
15 Finch, J.T., Lutter, L.C., Rhodes, D., Brown, R.S., Rushton, B., Levitt, M. and Klug, A. (1977) Nature 269, 29-36
16 Ansevin, A.T., Hnilica, L.S., Spelsberg, T.C. and Kelm, S.L. (1971) Biochemistry 10, 4793-4803
17 Li, H.J., Chang, C. and Weiskopf, M. (1973) Biochemistry 12, 1763-1772
18 Wilhelm, F.X., De Murcia, G.M., Champagne, M.H. and Daune, M.P. (1974) Eur. J. Biochem. 45, 431-443
19 Sahasrabuddhe, C.G. and van Holde, K.E. (1974) J. Biol. Chem. 249, 152-156
20 Woodcock, C.F.L. and Frado, L.L.Y. (1975) Biochem. Biophys. Res. Commun. 66, 403-410
21 Miller, P., Kendell, F. and Nicolini, C. (1976) Nucleic Acids Res. 3, 1875-1881
22 Mandel, R. and Fasman, G. (1976) Nucleic Acids Res. 3, 1839-1855
23 Whitlock, J.P. and Simpson, R.T. (1976) Nucleic Acids Research 3, 2255-2266
24 Lurquin, P.F. and Seligy, V.L. (1976) Chem.-Biol. Interactions 13, 27-45
25 Lawrence, J.J., Chan, D.C.F. and Piette, L.H. (1976) Nucleic Acids Res. 3, 2879-2893
26 Staynov, D.Z. (1976) Nature 264, 522-525
27 Olins, D.E., Bryan, P.N., Harrington, R.E., Hill, W.E. and Olins, A.L. (1977) Nucleic Acids Res. 4, 1911-1931
28 Lewis, P.N. (1977) Can. J. Biochem. 55, 736-746
29 Vengerov, Y.Y. and Popenkov, V.I. (1977) Nucleic Acids Res. 4, 3017-3027
30 Defer, N., Kitzis, A., Kruth, J., Brahms, S. and Brahms, J. (1977) J. Nucleic Acids Res. 4, 2293-2306
31 Lilley, D.M.J. and Tatchell, K. (1977) Nucleic Acids Res. 4, 2039-2055
32 Wittig, B. and Wittig, S. (1977) Nucleic Acids Res. 4, 3907-3917
33 Cotter, R.I. and Lilley, D.M.J. (1977) FEBS Letters 82, 63-68.
34 Stein, A., Stein-Bina, M. and Simpson, R.T. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 2780-2784
35 Garret, R.A. (1971) Biochemistry 10, 2227-2230
36 Woodcock, C.F.L. and Frado, L.-L.Y. (1976) J. Cell Biol. 70, 267a (Abst.)
37 Pardon, J.F., Worcester, D.L., Wooley, J.C., Cotter, R.I., Lilley, D.M. and Richards, B.M. (1977) Nucleic Acids Res. 4, 3199-3214.
38 Sollner-Webb, B. and Felsenfeld, G. (1975) Biochemistry 14, 2915-
39 Birnboim, H.C., Holford, R.M. and Seligy, V.L. Cold Spring Harbor Symp. Quant. Biol. 42 (in press)
40 Markov, G.G. and Ivanov, I.G. (1974) Anal. Biochem. 59, 555-563
41 Dahlberg, A.E., Dingman, C.W. and Peacock, A.C. (1969) J. Mol. Biol. 41, 139-147
42 Laemmli, U. (1970) Nature 227, 680-685
43 Panyim, S. and Chalkley, R. (1969) Biochemistry 8, 3972-3979
44 Tobin, R.S. and Seligy, V.L. (1975) J. Biol. Chem. 250, 358-364.

45 Gordon, C.N. and Kleinschmidt, A.K. (1968) Biochem. Biophys. Acta 155, 305-307
46 Olins, A.L., Cartson, R.D., Wright, E.B. and Olins, D.E. (1976) Nucleic Acids Res. 3, 3271-3291
47 Todd, R.D. and Garrard, W.T. (1977) J. Biol. Chem. 252, 4729-4738
48 Varshavsky, A.J., Bakayev, V.V., Chumackov, P.M. and Georgiev, G.P. (1976) Nucleic Acids Res. 3, 2101-2114
49 Bakayev, V.V., Bakayeva, T.G. and Varshavsky, A.J. (1977) Cell 11, 619-629
50 Kovacic, R.T. and van Holde, K.E. (1977) Biochemistry, 16, 1490-1498
51 Lohr, D., Corden, J., Tatchell, K., Kovacic, R.T. and van Holde, K.E. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 78-83.
52 Shelton, K.R. and Neelin, J.M. (1971) Biochemistry 10, 2342-2348
53 Seligy, V.L. and Miyagi, M. (1974) Eur. J. Biochem. 46, 259-269
54 Seligy, V.L., Poon, N.H., Dove, M. and Tobin, R.S. unpublished.
55 Sanders, M.M. and Hsu, J.T. (1977) Biochemistry 16, 1690-1695.
56 Campbell, A.M. and Cotter, R.I. (1977) Nucleic Acids Res. 4, 3877-3886
57 Li, H.J., Hu, A.W., Maciewicz, R.A., Cohen, P., Santella, R.M. and Cang, C. (1977) Nucleic Acids Res. 4, 3839-3854
58 Seligy, V.L. and Miyagi, M. (1969) Expt'l Cell Res. 58, 27-34.
59 Brasch, K., Setterfield, G. and Seligy, V.L. (1971) Expt'l Cell Res. 65, 61-72
60 Olins, D.E. and Olins, A.L. (1972) J. Cell Biol. 53, 715
61 Li, H.J. (1977) "Chromatin Structure - A Model" in The Molecular Biology of the Mammalian Genetic Apparatus, Ts'o, P., Ed., pp. 323-343. Elsevier/North-Holland Biomedical Press.
62 Woodcock, C.F.L. (1977) Science 195, 1350-1352
63 Camerini-Otevo, R.D. and Felsenfeld, G. (1977) Nucleic Acids Res. 4, 1159-1181
64 Weintraub, H., Worcel, A. and Alberts, B. (1976) Cell 9, 409-417
65 Weintraub, H. and van Lente, F. (1974) Proc. Nat. Acad. Sci. U.S.A. 71, 4249-4253
66 Oudet, P., Spadafora, C. and Chambon, P. Cold Spring Harbor Symp. Quant. Biol. 42 (in press)
67 Polacow, I., Cabasso, L. and Li, H.J. (1976) Biochemistry 15, 4560-4565
68 Weischet, W., Tatchell, K., Van Holde, K. and Klump, H. (1978) Nucleic Acids Res. 5, 139-160.