Effects of Various Salts and pH on the Stability of the Nucleosome in Chromatin Fragments†

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ABSTRACT: The stability of nucleosomes in long chromatin fragments was observed by differential scanning calorimetry over a wide range of solution conditions. The thermal denaturation of chromatin was characterized in general as three major transitions, although the process clearly is more complex. The three major transitions were (1) denaturation of the nucleosome, (2) base unstacking of DNA in the resulting denatured nucleoprotein, and (3) base unstacking of naked DNA. In very low salt concentrations (e.g., 2 mM sodium cacodylate), these three processes were essentially coincident (near 76 °C), but in medium salt concentrations (e.g., 100 mM NaCl) the nucleosome denaturation occurred first at about 69 °C and then base unstacking occurred at 85 °C. As [NaCl] was increased, all three processes were resolved with the observation of increasing amounts of naked DNA being melted, until at 2000 mM NaCl the calorimetric profile showed mainly the melting of DNA. The transition temperature for nucleosome denaturation decreased from 76 to 63 °C as the salt concentration increased from 1 to 600 mM. Destabilization of the nucleosome by increasing [NaCl] was also evident above 100 mM as a decrease in enthalpic change attributable to nucleosome denaturation. Similarly, as [NaCl] was increased above 100 mM, less and less denatured nucleoprotein was evident as more and more of the DNA melted as naked DNA. The fatty acid salts, sodium valerate and sodium caproate, destabilized the nucleosome but not the denatured nucleoprotein that resulted from the collapse of the nucleosome. In the series acetate, butyrate, valerate, caproate, it was clear that destabilization of the nucleosome increased as hydrophobicity (chain length) increased. Pimelate, with the same number of carbon atoms as caproate but with an extra negative charge, did not destabilize the nucleosome as caproate did. The nucleosome was substantially stabilized by $MgCl_2$ within the range 0.5–2 mM and by spermidine in the range 0.1–3 mM. The transition temperature for DNA in the denatured nucleoprotein was unaffected by spermidine, but was lowered by $MgCl_2$. Spermidine was more effective than $MgCl_2$ at displacing DNA from the denatured nucleoprotein.

Previously, we reported (Jin & Cole, 1986; Guo & Cole 1989a,b) that the condensation of chromatin was strongly affected by particular ions and by pH in the physiological range. This made clear the importance of paying attention to buffer composition when comparing reports of chromatin function and dynamics. Such factors might also have substantial effects on the structure of the nucleosome when it is contained within chromatin, but only modest attention seems to have been given to that possibility. The work to be presented here was an exploration of salt and pH effects on the stability of the nucleosome in large fragments of chromatin. The technique used to observe stability was differential scanning calorimetry because it is applicable to chromatin even in the physiological range of pH and ionic conditions where precipitation occurs.

Studies of chromatin at salt concentrations below 10 mM by differential scanning calorimetry (Reczek et al., 1982; Riehm & Harrington, 1987) or other thermal denaturations have been reported before, as well as similar studies on core particles (Weischet et al., 1978; Simpson & Shindo, 1979; Bryan et al., 1979; Bina et al., 1980; Cowman & Fasman, 1980; McGhee & Felsenfeld, 1980), nucleosomes (Simpson, 1978), and whole nuclei. At higher salt concentrations, in the physiological range, we investigated whole nuclei (Touchette & Cole, 1992) and chromatin (Almagor & Cole, 1989). Our earlier studies on isolated chromatin revealed two major structural transitions as the temperature was raised from 25 to 95 °C. The first transition was clearly shown to be the collapse of the nucleosome. The second transition was at the temperature of base unstacking of naked, relaxed DNA, but as was pointed out then, it was not clear whether the DNA was free or still bound to proteins. The present results clarified that issue by using a wide range of ionic conditions, to which nucleoprotein and naked DNA responded differently.

METHODS AND MATERIALS

Cell Culture. HeLa cells, strain S3, were maintained in suspension culture at cell densities of (2–8) × 10^5/mL in Joklik's modified spinner medium supplemented with 5% calf serum. Cells were harvested at cell densities of (5–6) × 10^5/mL.

Isolation of Nuclei. All steps were done at 0–4 °C, and all centrifugation was at 300*g* for 3 min. HeLa cells were pelleted and washed in swelling buffer (0.1 M hexylene glycol, 1 mM $CaCl_2$, and 0.06 mM PIPES,[1] pH 6.8) with 0.25 mM PMSF, which was added just before use. Then the cells were resuspended in swelling buffer with 1 mM PMSF for 10–15 min at a density of (1.3–2.6) × 10^7/mL. Cells were disrupted with 10 strokes of a loose-fitting Dounce homogenizer. The nuclei were washed three times in the same swelling buffer, two times in buffer A (50 mM Tris, 25 mM KCl, 0.3 M

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[1] Abbreviations: PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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sucrose, and 5 mM $MgCl_2$, pH 6.5) with 0.1% (v/v) Triton X-100 (Boehringer, purified for membrane research), and three times in buffer A without Triton. All washing buffers contained 1 mM PMSF, which was added just before use. The water used was Milli-Q water from Ultra-Pure Water System (resistance above 15 MΩ·cm).

Isolation of Chromatin. For large chromatin fragments, nuclei were resuspended in buffer A with 1 mM PMSF at A_{260} = 150. After preincubation at 37 °C for 5 min, micrococcal nuclease (Worthington) was added at 200 units/mL and $CaCl_2$ was added to 1 mM for additional incubation at 37 °C for 225 s. The reaction was quenched with cold 200 mM EDTA (pH 6.5) (final concentration, 10 mM) and kept on ice. The pellets were resuspended in 2 mM EDTA (pH 7.0) on ice and allowed to lyse for 2 h, with occasional gentle mixing. Chromatin was recovered in the supernatant after centrifugation, and its concentration was measured assuming A_{260} = 270 for 1% DNA in 0.1 M NaOH. The solution was stored at −80 °C.

For medium or short chromatin fragments, the procedure was the same except that nuclease digestion was done at an A_{260} of 100, and the enzyme incubation was micrococcal nuclease of 100 units/mL. Digestion was for 30 or 60 min for medium or short fragments, respectively.

Extraction of DNA and DNA Electrophoresis. Chromatin samples diluted 6-fold were deproteinized in an aqueous phase containing 30 mM Tris (pH 7.8), 1% SDS, 0.01% proteinase K, and 1 M NaCl and then extracted twice with equal volumes of chloroform/isoamyl alcohol (24:1, v/v). The DNA was precipitated for 30 min in 2.5 vol of ethanol at −20 °C, centrifuged at 23000*g* for 15 min, and washed with 80% cold ethanol. The DNA was dried under vacuum for 10 min and then dissolved in electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, and 1 mM Na_2EDTA, pH 7.2) to make the DNA concentration 5 μg/μL. DNA fragments were separated in electrophoresis buffer on 2%, 1.5%, and 0.75% (w/v) agarose gels for short, medium, and long sizes, respectively. The gels were stained with ethidium bromide and photographed. Alternatively, the DNA gels were stained with Stains-All (Kodak) solution and scanned with an LKB 2202 ultroscan laser densitometer. For the Stains-All procedure, it is necessary to do pre-electrophoresis; 54 V for 3 h before loading the DNA samples. Relative amounts of fragments of different size were determined from the areas of peaks observed by scanning.

Typical preparations of long, medium, and short chromatin fragments are represented, respectively, by Figure 1A,B,C. The percentages of fragments that were pentamers of nucleosomes or shorter were 14% for Figure 1A, 40% for Figure 1B, and 76% for Figure 1C.

Naked DNA used for calorimetry was prepared from chromatin by phenol extraction as above but scaled up, and the DNA was finally dissolved in water instead of electrophoresis buffer.

Protein Electrophoresis. Chromatin samples were analyzed for histones by 13.5% SDS–polyacrylamide gel electrophoresis, essentially according to Laemmli (1970). Quantitative measurement of histones was done by scanning densitometry of Coomassie-stained gels using color factors for the individual histones as published previously (Ring & Cole, 1979). The amount of H1 histone ranged from 0.75 to 0.88 molecules per octamer of core histones.

Differential Scanning Calorimetry. Samples used for calorimetry were dialyzed against buffer (15 mM PIPES and 1.5 mM NaN_3, pH 7.0) at 4 °C for 4 h. The volume ratio

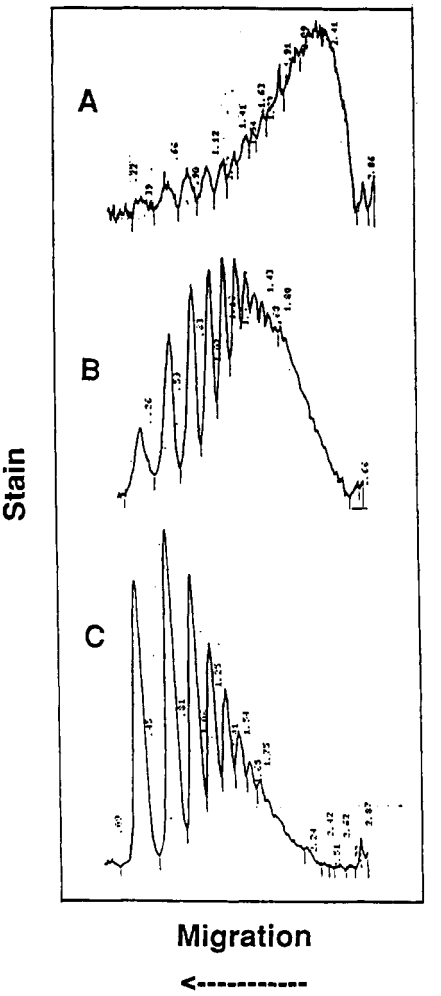

FIGURE 1: Scanning densitometry of electrophoretic gels of chromatin preparations containing fragments of different sizes. The first peak on the left represents mononucleosomes, as confirmed by size markers run in parallel.

of chromatin sample to buffer was about 1:200. PMSF (100 mM) was added to a final concentration of 0.5 mM in the beginning, and the same volume was added once more after 2 h of dialysis. For use in the reference cell of the calorimeter, some 2 mM EDTA solution (pH 7.0) was dialyzed alongside the sample of chromatin. After dialysis, chromatin samples and reference solutions were stored at −80 °C.

Scanning calorimetry experiments were run on an MC-2 ultrasensitive scanning calorimeter (MicroCal, Inc.) from 20 to 107 °C at a scanning rate of 20 °C/h. Output was recorded by computer and recorder (Model 2000, Bausch & Lomb). Prior to scanning, samples and reference solutions were defrosted on ice and degassed under vacuum for 15 min at room temperature. Degassed 1.50 or 5.00 M NaCl stock solution, pH 7.0, was added to specified final concentrations, mixed by Pasteur pipette, and then loaded into the calorimetric cells. The sample amount ranged from 0.928 to 2.28 mg of DNA in 1.20 mL, the volume of the calorimeter cell.

In some experiments, the following stock solutions were used instead of the stock sodium chloride solution: acetate, 1.50 or 3.00 M; *n*-butyrate, 3.00 M; *n*-valerate, 1.50 or 3.24 M; *n*-caproate, 2.18 M; pimelate, 2.38 M; phosphate, 0.753 M. All were sodium salts at pH 7.0. Spermidine (8.78 mM, pH 7.0) and magnesium chloride (20 mM) were used also. In still other experiments, chromatin samples were dialyzed to 1.0 mM sodium cacodylate (pH 7.0) in the absence of all other additives listed above. Sodium cacodylate (100 mM,

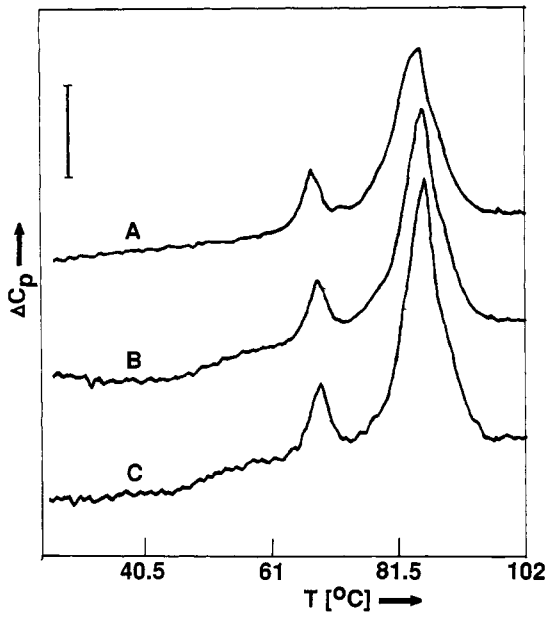

FIGURE 2: Differential scanning calorimetry of chromatin fragments of different lengths. All profiles represent the thermal denaturation of chromatin in 100 mM NaCl, 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF, pH 7.0, at 20 °C: A, short fragments (see text); B, medium fragments; C, long fragments.

pH 7.0) was used to adjust samples containing cacodylate from 1.0 to 10 mM.

The raw data were normalized to millicalories per degree per gram of DNA by computer with the baseline subtracted. The vertical bar equals 1 mcal/deg/g in all profiles shown. For deconvolution, the data between about 50 and 100 °C were usually used, depending somewhat on solution conditions. The DECONV program was used, assuming independent non-two-state transitions with the calorimetric heat, $\Delta H°$, unequal to the van't Hoff heat, ΔH^*. Results are reported as calorimetric heat or enthalpy change at constant pressure, $\Delta H°/g$ of DNA.

RESULTS

Search for the Transition of the 30-nm Fiber of Chromatin. Initially, we had hoped to detect a structural transition of the 30-nm-fiber compacted form (van Holde, 1989) of chromatin by the comparison of melting profiles at 20 and 100 mM NaCl since 30-nm fibers are formed at the latter concentration but not at the former. In addition, we compared chromatin fragments that were too short to form 30-nm fibers with larger fragments, under solution conditions known (Thoma et al., 1979) to induce the compaction of long chromatin fragments into the 30-nm form. Three kinds of chromatin preparations were submitted to scanning calorimetry in this series. In the first of these preparations, 76% of the fragments contained five or fewer nucleosomes and therefore could not form 30-nm fibers under any conditions. The other two preparations were of medium size, where 60% of the DNA was between 1.2 and 2.1 kbp, or of long size, where 85% of the DNA was from 1.2 to 6.0 kbp. As may be observed in Figure 2, no detectable signal was missing when the short fragments were compared to longer ones. In every case two major structural transitions were evident: one for the nucleosome and a second one for base unstacking of DNA whether still complexed to proteins or not. In the case of the short fragments, the transitions were slightly broader and occurred at slightly lower temperatures than the corresponding transitions for longer fragments, but there was no evidence of a transition for the 30-nm fiber.

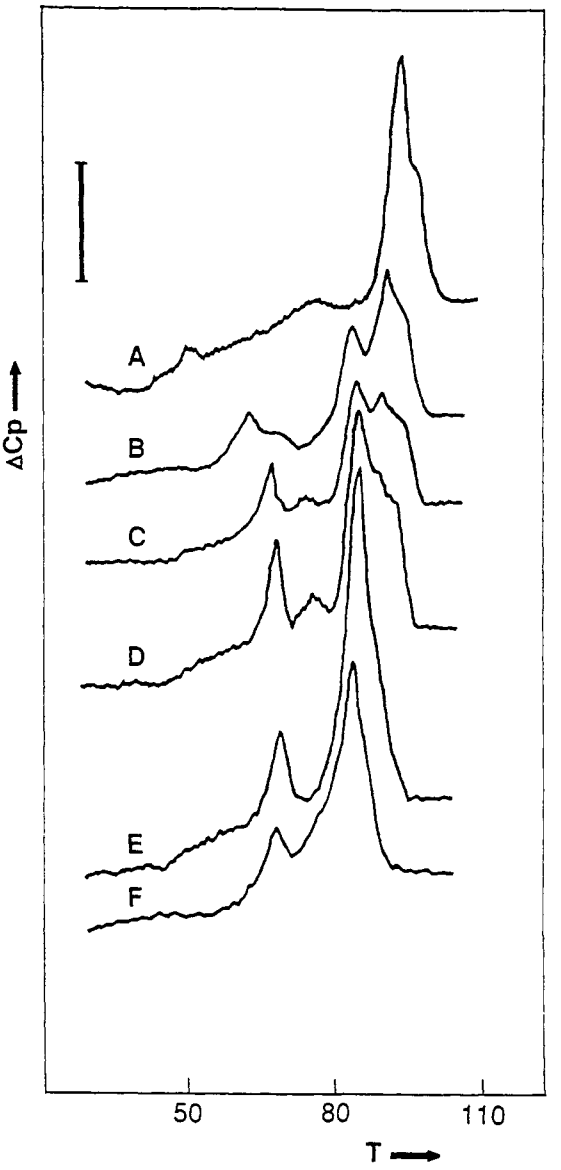

FIGURE 3: Effect of NaCl on thermal denaturation of chromatin. All buffers were 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF, pH 7.0. [NaCl]: A, 2000 mM; B, 600 mM; C, 450 mM; D, 300 mM; E, 100 mM; F, 20 mM.

The comparison of conditions that induce 30-nm fibers to solution conditions that do not also failed to reveal a substantial calorimetric signal that could be attributed to the 30-nm fiber. The scanning calorimetry of long chromatin fragments at 100 mM NaCl (Figure 3E) contained no features in addition to those observed for long fragments at 20 mM NaCl (Figure 3F). Most of the profiles shown in Figure 3 were replicated with both medium- and long-type chromatin preparations, and it was clear that fragment size did not affect the profiles.

To learn whether a signal for the structural transition of the 30-nm fiber had been lost by increasing the temperature too rapidly, the scanning rate was varied from 10 to 90 deg/min. Although at the upper end of this range melting temperatures were slightly elevated (1.5 °C), the profiles all looked alike. We can conclude that the heat capacity of the compacted, 30-nm-fiber form of chromatin is too small to be detected or that size polydispersity causes such compacted fragments to unwind over a broad range of temperatures.

Effects of NaCl on Nucleosome Stability. A series of calorimetric scans of long chromatin fragments was obtained

Table 1: Salt Effects on the Thermal Transitions of Chromatin[a]

salt	concentration (mM)	T_m (°C) Nu	T_m (°C) DNP	$\Delta H°$ (cal/g) Nu	$\Delta H°$ (cal/g) total
NaCl	2000	50.2	75.6		15.2
	600	62.9	84.1	2.66	18.4
	450	67.2	84.7	2.45	19.5
	300	68.2	85.4	2.89	22.9
	100	68.9	85.8	3.11	24.4
	20	68.5	84.2	3.02	19.7
cacodylate	10	73.4	84.2	4.26	14.6
	5	72.1[b]	82.2	4.21[b]	17.2
	2		75.1		11.8
	1		74.2		11.5
	1[c]		72.9		12.3

[a] T_m gives the transition temperature and $\Delta H°$ the enthalpic change for the nucleosome (Nu) and for the denatured nucleoprotein (DNP) derived from the denaturation of the nucleosome. [b] The early melting region is complex and does not show a clear peak for the nucleosome, which could be taken to have melted between 68 and 75 °C. The average over this temperature span is shown for T_m and $\Delta H°$. [c] In addition to 1 mM cacodylate, this sample contained 2 mM EDTA.

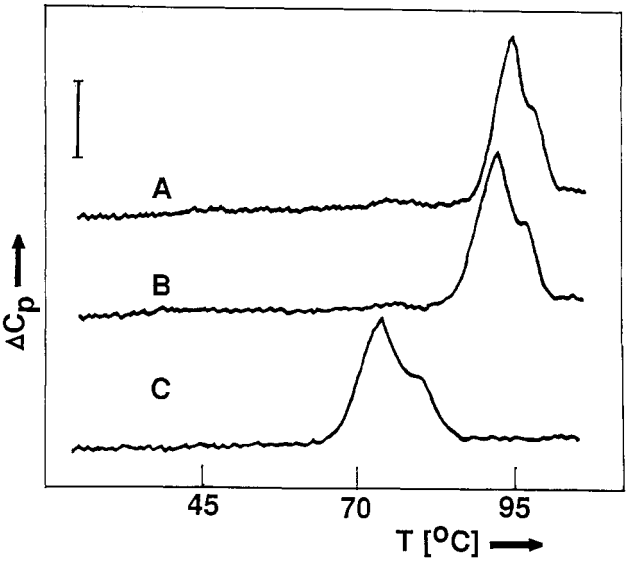

FIGURE 4: Scanning calorimetry of naked DNA. Buffers were 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF, pH 7.0, with NaCl added to A, 2000 mM; B, 600 mM; C, 20 mM.

Table 2: Effects of Ions on the Thermal Transitions of Naked DNA[a]

ions	concentration (mM)	T_m (°C)	$\Delta H°$ (cal/g)
NaCl	2000	94.3	14.6
	600	92.2	15.5
	300	89.2	15.3
	100	82.7	15.7
	20	74.1	15.6
valerate	600	87.2	15.3
	300	86.3	15.8
	200	84.7	15.8
	50	78.1	14.9
spermidine	0.1	70.8	15.5
phosphate	150	87.9	15.6

[a] Abbreviations same as in Table 1.

at several concentrations of NaCl (Figure 3 and Table 1), and a parallel series was done for naked DNA (Figure 4 and Table 2). In describing the calorimetric profiles, we will consider three general components, which can be most easily recognized as the three major peaks in curve C of Figure 3. The transition near 67.2 °C in this profile is taken to be the denaturation of nucleosomes for reasons previously published (Almagor & Cole, 1989) and because, as will be shown here, it is stabilized by hydrophobic forces as well as electrostatic ones. Both the transition at 67.2 °C and the one at 84.7 °C disappeared when the addition of NaCl to 2000 mM dissociated DNA and histones, and so both of these transitions must be due to nucleoproteins. The 84.7 °C transition most likely represents denatured nucleoprotein derived from the collapse of the nucleosome since (see following) its dependence on concentrations of NaCl and fatty acid salts shows it to be stabilized by electrostatic forces but not by hydrophobic ones. The third major transition, seen as the highest melting general component in Figure 3 (curve C), evidently is due to naked DNA since the corresponding transition gives the only major peak after disruption of all of the nucleoprotein at 2000 mM NaCl (Figure 3, curve A).

Between 20 and 450 mM NaCl, the temperature for the denaturation of the nucleosome dropped gradually from 69 to 67 °C, while the melting of the nucleoprotein derived from denaturation of the nucleosome occurred at about 85 °C with little dependence on salt concentration (Table 1). At 600 mM for nucleosomes and at 2000 mM for the denatured nucleoprotein the transition temperatures dropped drastically. In fact, at 2000 mM NaCl, where histones are known to be completely displaced from chromatin, the only major peak in the scanning profile essentially looks like that of naked DNA (compare Figure 3 with Figure 4).

In our previous work (Almagor & Cole, 1989), it was not clear whether the melting of the denatured chromatin derived from the collapse of the nucleosome represented free DNA or DNA with denatured proteins bound to it. Inspection of the series of scans in Figure 3 taken for chromatin at salt concentrations from 20 to 2000 mM NaCl and for naked DNA over a similar range of salt concentrations (Figure 4) makes it evident that, under the conditions (100 mM NaCl) used in our previous work, much and perhaps most of the denatured chromatin derived from the collapse of the nucleosome melted near 85 °C as a nucleoprotein. In the series of profiles observed as NaCl was increased above 100 mM, however, a complex shoulder grew on the high-temperature side of the 85 °C transition. The shoulder grew at the expense of the 85 °C peak until at 2000 mM NaCl the original peak for denatured nucleoprotein had disappeared and the only defined transition exhibited the same position, the same shape, and the same magnitude ($\Delta H°$) as free DNA. Our interpretation is that at low salt concentrations the melting of the nucleosome was followed by the melting of nucleoprotein and that increasing proportions of the denatured chromatin melted as free DNA as the salt concentration was raised.

Visual inspection of the melting profiles of naked DNA reveals that a minimum of three curves would be necessary for deconvolution (Figure 5). Presumably, the three deconvoluted peaks represent bulk DNA flanked by lower melting AT-rich satellites and higher melting GC-rich satellite DNA. The actual situation, of course, is essentially a continuum of different fragment sizes of different base compositions, but deconvolution into three component curves gives a good mathematical fit to the observed overall profile. Even with the oversimplification of using three curves for naked DNA, however, it became clear that deconvolution of the major part of the scanning profile for chromatin led to ambiguous results. For example, Figure 5 shows that a well-fitting deconvolution of curve B from Figure 3 required a component for the nucleosome, another for a transition near 70 °C, two for the main part of the denatured nucleoprotein, and three for naked DNA. Testing our calculations, we found that with such a large number of components, good fits could be obtained with

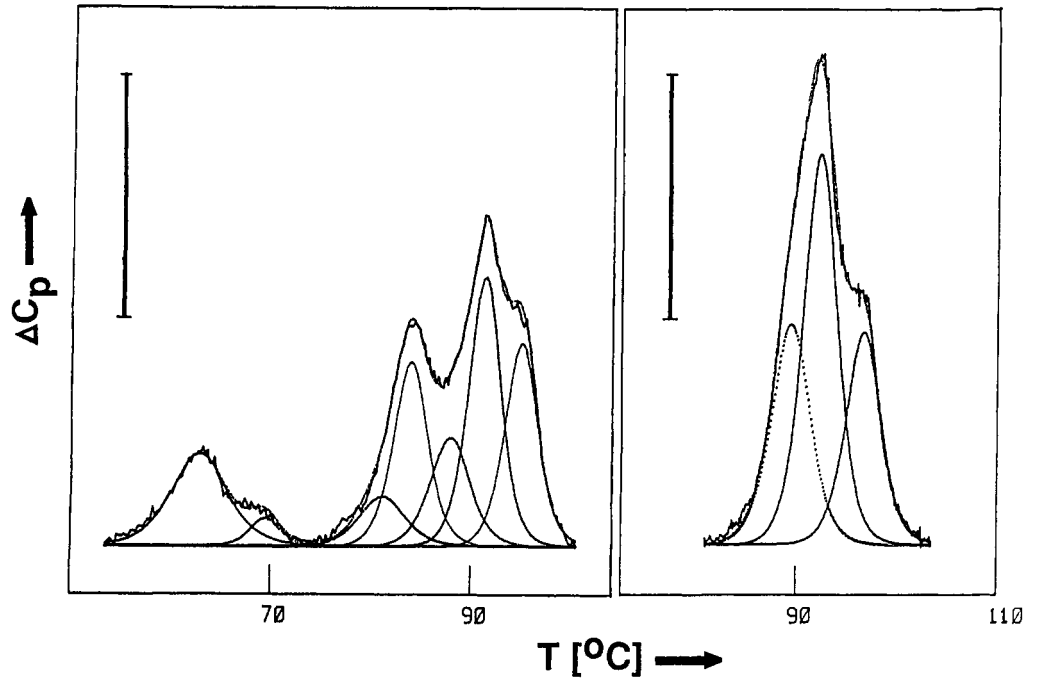

FIGURE 5: Examples of deconvolution. Scanning was in 600 mM NaCl, 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF, pH 7.0, for chromatin (left) and naked DNA (right). The curve representing the summation of component curves is superimposed on the observed curve and is scarcely visible.

Table 3: Effects of Fatty Acid Salts on the Thermal Transitions of Chromatin[a]

		T_m (°C)		$\Delta H°$ (cal/g)	
anion	concentration (mM)	Nu	DNP	Nu	total
valerate	600		86.8		17.9
valerate	300	61.2	84.9	2.17	19.3
valerate	200	64.1	85.2	2.22	19.9
valerate	50	68.5	84.8	2.43	18.5
acetate	200	68.9	86.2	2.72	20.4
butyrate	200	67.5	85.9	2.63	22.2
caproate	200	50.9	83.7	2.05	25.2
pimelate	100	68.3	85.9	2.63	22.2

[a] Abbreviations same as in Table 1.

Table 4: Effects of Various Ions on the Thermal Transitions of Chromatin[a]

		T_m (°C)		$\Delta H°$ (cal/g)	
ion	concentration (mM)	Nu	DNP	Nu	total
phosphate	150	67.4	84.7	2.17	20.6
	15	68.2	84.4	2.68	19.2
$MgCl_2$	2	72.8	81.3	5.18	23.5
	1	70.6	83.7	4.33	19.1
spermidine	3	75.3	84.3	3.89	14.9
	1	73.9	83.8	5.29	21.5
	0.1	71.1	83.9	5.16	20.4

[a] Abbreviations same as in Table 1.

multiple combinations of assumed positions and magnitudes of the components. Therefore, we feel it is not justified to interpret the profiles of chromatin melting at a level of precision beyond what is clear in simple visual inspection. In most cases, the denaturation of the nucleosome was resolved well enough that reasonable estimates of its $\Delta H°$ could be made by deconvolution, and these are listed in Tables 1, 3, and 4. Estimates were made of $\Delta H°$ for nucleosome denaturation and of total $\Delta H°$ for nucleosome plus denatured nucleoprotein plus naked DNA by drawing a baseline from the start of the nucleosome transition to the finish of the nucleoprotein or naked DNA, whichever was later. This omitted from the

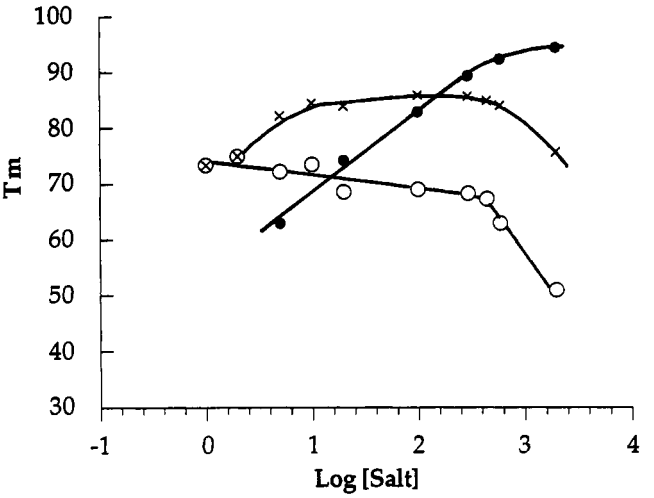

FIGURE 6: Comparison of the effects of salt on principal structural transitions. Symbols: ○, nucleosome; ×, denatured nucleoprotein; ●, naked DNA. Buffers at 20 mM and higher were 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF with varying amounts of NaCl at the concentrations shown on the abscissa. Buffers at 10 mM and lower were 1 mM PMSF and cacodylate (pH 7.0) at the concentration shown.

calculation a broad spread of minor thermal processes (Figure 3) that we cannot identify. These processes may represent non-histone chromosomal proteins and in some cases dissociated histones or unknown structures. In any case, this ill-defined part of our thermal profiles is much too complex to be included in our analysis.

The effect of NaCl on the stability of various chromatin components is evident in Figures 3 and 6. In terms of transition temperatures, salt destabilized the denatured nucleoprotein and destabilized the nucleosome even more so. In contrast, naked DNA was stabilized as [NaCl] was increased, as would be expected. The observed enthalpic change in melting naked DNA was constant over the range 20–2000 mM NaCl at 15.7 ± 0.26 cal/g of DNA (Table 2). Over this range of salt concentrations, the change in enthalpy observed for the thermal

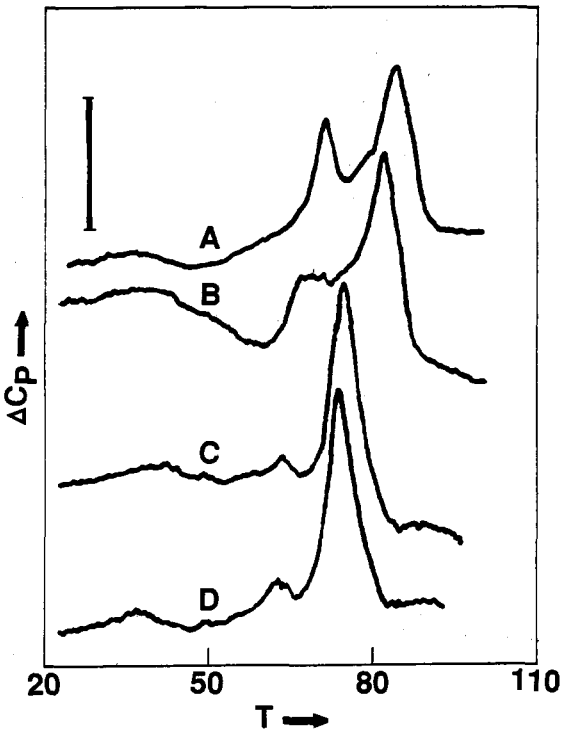

FIGURE 7: Effect of low concentrations of salt on thermal denaturation of chromatin. Buffers at pH 7.0 all contained 1 mM PMSF and (A) 10 mM, (B) 5 mM, (C) 2 mM, or (D) 1 mM sodium cacodylate.

transition of chromatin from the start of the denaturation of the nucleosome to the final unstacking of bases in denatured nucleoprotein and DNA was consistently higher than the figure for naked DNA alone. The $\Delta H°$ for chromatin was maximal at 24.4 cal/g of DNA in 100 mM NaCl and descended to 18.4 cal/g of DNA in 600 mM NaCl and to 21.1 cal/g of DNA at 20 mM NaCl.

Seen most clearly at 76 °C in the scan at 300 mM NaCl (Figure 3D), there was a minor transition between the denaturation of the nucleosome and the major melting of DNA in denatured nucleoprotein. At lower salt concentrations, it appeared as a shoulder on the low-temperature side of the main peak for denatured nucleoprotein. Whatever structure it represents was severely destabilized by NaCl compared to the other components detectable in these profiles. By the time the salt concentration was increased to 600 mM, this minor transition was found as a shoulder on the high-temperature side of the peak for nucleosome denaturation. For the present, at least, the identity of this minor transition can only be guessed. It might represent a special class of nucleosomes containing modified histones or some non-histone proteins or sequence variants of histones. There are other possibilities of course, and its identification must await further study.

Effects of Low Salt Concentrations. To study the stability of nucleosomes in chromatin fragments at low salt concentrations, where essentially all previous investigations were conducted, we used a series of cacodylate concentrations from 1 to 10 mM. The calorimetric scans obtained from this study are shown in Figure 7 (see also Figure 6 and Table 1). The profile for chromatin at 1 mM cacocylate looks much like the profile for core particles observed by Weischet et al. (1978) under the same conditions. Our profiles for chromatin at 1 and 2 mM cacodylate also look like those reported for core particles in similar concentrations of other salts (Bryan et al., 1979; Simpson, 1978; Simpson & Shindo, 1979; Bina et al., 1980; McGhee & Felsenfeld, 1980; Cowman & Fasman, 1980). For example, Weischet et al. found a premelt at ca. 61 °C and a major transition at 74.3 °C. Weischet et al. also showed that for core particles the denaturation of the nucleosome and base unstacking were nearly coincident at 74 °C, as seems to be the case with the present observations on chromatin at 1 and 2 mM. In the present work, it can be seen that at 5 or 10 mM, however, the nucleoprotein that was derived from nucleosome denaturation in chromatin was stable enough to delay base unstacking until the temperature was a few degrees above that of nucleosome denaturation.

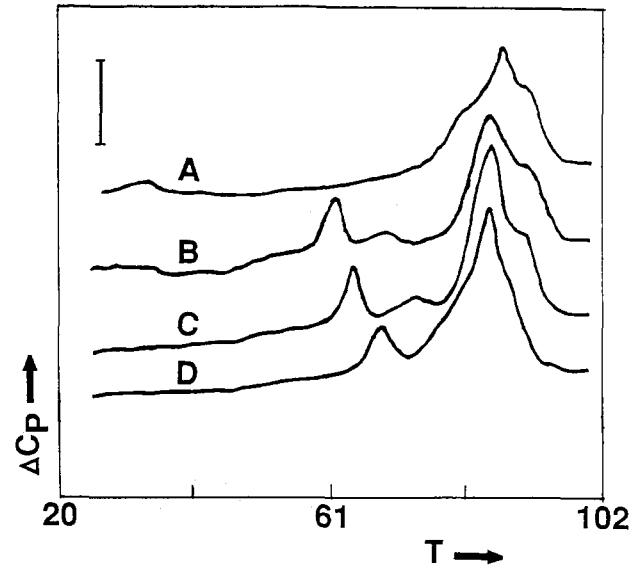

FIGURE 8: Effect of sodium valerate on the thermal denaturation of chromatin. Buffers were 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF, pH 7.0, with valerate added to A, 600 mM; B, 300 mM; C, 200 mM; or D, 50 mM.

The first thermal transition, seen by Weischet et al. at 61 °C for core particles, was shown to be due to DNA at the ends of the core particle. Although at first glance a somewhat similar interpretation might seem to apply to the transition we found at 63 °C for chromatin, it ought to be noted that for our rather large chromatin fragments, only 9% of the total enthalpic change was observed in the 63 °C transition compared to 27% in the case of the 61 °C transition of core particles.

Effects of Fatty Acid Salts. Having previously observed (Guo & Cole, 1989b) selective effects of fatty acid salts on chromatin condensation, we examined their effects on nucleosome stability. A series of calorimetric scans for chromatin at various concentrations of sodium valerate is presented in Figure 8 (see also Table 3. As in the case of NaCl, increasing concentrations of valerate destabilized the nucleosome and the derived nucleoprotein while increasing the stability of free DNA. The fatty acid salt was less effective than NaCl (on an equimolar basis) in the stabilization of free DNA, but substantially more effective than NaCl in destabilizing the nucleosome. No significant difference was evident between NaCl and sodium valerate in their effects on the denatured nucleoprotein.

Other fatty acid salts were compared to valerate at 200 mM concentrations. The data summarized in Table 3 make it clear that the length of the carbon chain is a significant factor in the destabilization of the nucleosome, but not in the case of denatured nucleoprotein. Destabilization of the nucleosome by 200 mM caproate was almost as dramatic as it was in 600 mM valerate, and neither of these conditions had a large effect on the transition of the denatured nucleoprotein.

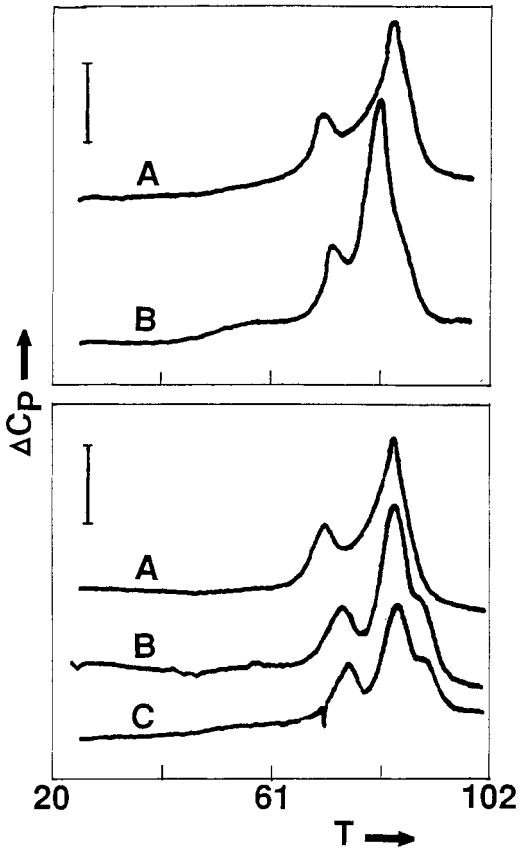

FIGURE 9: Effect of $MgCl_2$ and spermidine on the thermal denaturation of chromatin. Upper panel: A, 0.5 mM $MgCl_2$; B, 2.0 mM $MgCl_2$. Lower panel: A, 0.1 mM spermidine; B, 1 mM spermidine; C, 3 mM spermidine. In all cases buffers were 15 mM PIPES, 1.5 mM NaN_3, and 1 mM PMSF, pH 7.0.

Not surprisingly, the hydrophobic factor in extending the carbon chain could be offset by increasing the number of ionic charges on the organic acid. The dicarboxylate ion pimelate, with the same number of carbons as caproate, gave the same nucleosome stability as did NaCl or sodium acetate, while caproate was much more destructive toward the nucleosome.

Effects of Tightly Bound Cations and Sodium Phosphate. Previous studies on the condensation of chromatin revealed a significant difference between Mg^{2+} and Na^+ in their effects (Ausio et al., 1984b; Jin & Cole, 1986), and spermidine was also shown (Jin and Cole, 1986) to differ from Na^+ in this regard. Calorimetric profiles are shown in Figure 9 for chromatin in different concentrations of $MgCl_2$ and spermidine. Even at as low a concentration as 0.5 mM, $MgCl_2$ stabilized the nucleosome compared to 100 mM NaCl, and increasing its concentration to 2 mM increased the melting temperature and the enthalpic change even more (Table 4). In contrast, the $MgCl_2$ destabilized the denatured nucleoprotein, and it did so without substantially converting the nucleoprotein to free DNA. Like Mg^{2+}, spermidine stabilized the nucleosome but it was even more powerful. Unlike Mg^{2+}, however, spermidine had little effect on the melting temperature of the denatured nucleoprotein, and even at 1 mM it evidently converted much of the denatured nucleoprotein to free DNA.

Although pH has a dramatic effect on the condensation (Guo & Cole, 1989a) of chromatin in the physiological range, its effect on calorimetric scans was modest. The stability of both the nucleosome and the denatured nucleoprotein in 100 mM NaCl seemed indifferent to pH from 8.0 to 6.5, but the nucleosome was clearly destabilized at pH 5.5. The transition temperature for the nucleosome, which held at 68.2 °C from 8.0 to 6.5, dropped to 65.6 °C at pH 6.1 and to 62.0 °C at pH 5.5. The denatured nucleoprotein was less sensitive, showing a T_m of 85.9 °C above pH 6.1, a T_m of 84.8 °C at pH 6.1, and a T_m of 82.8 °C at pH 5.5. The destabilization of chromatin upon lowering the pH to 5.5 proved to be reversible. A chromatin sample dialyzed for 4 h to pH 5.5 revealed the destabilization of the nucleosome, but a similar sample dialyzed for 4 h to pH 5.5 and then for 4 h back to pH 7.0 gave a scanning profile like that of a sample that had remained at pH 7.0. This was expected since we had previously shown (Touchette & Cole, 1992) that chromatin contained within isolated nuclei having been exposed to pH 3.0 gave the same calorimetric scan at pH 7.0 as a sample that had not been exposed to an acidic pH.

The effect of phosphate was compared to that of chloride, with the results given in Table 4. The calorimetric profiles at 15 and 150 mM phosphate looked approximately like those for 20 and 300 mM NaCl, respectively (pH 7.0 in both cases), with regard to melting temperatures. Phosphate had little or no effect on the enthalpic changes.

DISCUSSION

Previously, core particles were reported (Weischet et al., 1978) to melt in two transitions in low salt concentrations; the smaller, first transition was interpreted as the base unstacking of 20 bp of DNA on either end of the core particle, and the larger, second transition was taken to be the base unstacking of the inner 100 bp of the DNA along with essentially coincident conformational changes in the DNA and the histone core. That interpretation of the major transition of core particles is directly applicable to the major transition of chromatin fragments in 1 mM cacodylate (Figure 7), but the nature of the first transition of chromatin fragments is not necessarily as simply related to the first transition of core particles. Yau et al., (1982) considered the DNA of chromatin in terms of four domains: (1) the 100-bp, central core DNA of the nucleosome; (2) the 20 bp of flanking DNA that lies on each side of the central core, completing the core particle; (3) the next 14 bp of loose core DNA on each side of the chromatosome; and (4) the linker DNA (13 bp in the case of HeLa cells) that extends beyond the chromatosome on both sides. The notion of such domains can be adapted to an interpretation of our calorimetric studies.

The magnitude of the first transition we observed for long chromatin fragments in 1 mM cacodylate is compatible with the melting of either loose core DNA or, more likely, linker DNA. It is also possible that it represents about 50 bp at each end of every fragment, but we favor the notion that the first transiton was the melting of linker DNA. The attribution of the first transition to linker DNA would agree with the conclusions of Reczek et al. (1982) and Riehm and Harrington (1987) in their studies of HeLa chromatin fragments in 5 mM sodium phosphate and 0.2 mM EDTA.

Further comparison of our results to those of Reczek et al. (1982) and Riehm and Harrington (1987) presents some difficulties. At 5 mM cacodylate our data can be deconvoluted to three transitions in good quantitative agreement with those of Riehm and Harrington, even though those authors used a different buffer, phosphate/EDTA at 5 mM. Reczek et al., however, report transitions at significantly lower temperatures than those of Riehm and Harrington and ours and substantially different enthalpic changes, despite their use of buffer conditions identical to those of Riehm and Harrington. The difference in enthalpic magnitudes may be partly due to uncertainties inherent in the deconvolution of complex profiles,

but the discrepancies in this case clearly go beyond that. Perhaps the discrepancies reflect differences in the preparation of chromatin. Compared to our results in Figure 7 at buffer concentrations below 5 mM, Riehm and Harrington and Reczek et al. found more DNA melting in the lower temperature transitions. Our calorimetric scans for chromatin fragments in 1 and 2 mM cacodylate indicated that, following the melting of linker DNA, all of the DNA of the chromatosome melted at the same time along with the denaturation of the histone core. Above about 5 mM salt, the DNA in denatured nucleoprotein was sufficiently stabilized by the salt to melt at a temperature distinctly higher than that of the denaturation of the nucleosome.

Over a wide range of salt concentrations, 20–600 mM, nucleoprotein remained after the denaturation of the nucleosome and base unstacking occurred while proteins were still bound to the DNA. Although histones clearly stabilize DNA in salt concentrations below about 10 mM, it was somewhat surprising that above approximately 150 mM salt the bound proteins actually destabilized the DNA, causing the nucleotide strands to separate at lower temperatures than they would in the case of naked DNA. At 2000 mM salt, the melting of DNA within denatured nucleoprotein was not seen, because the proteins were largely displaced from the DNA before the start of the calorimetric scan. Below about 5 mM salt, where naked DNA is less stable than the nucleosome, the denaturation of the nucleosome did not produce a distinct nucleoprotein, but led immediately to base unstacking of the DNA.

Dissociation of DNA from core particles has been observed frequently (Cary et al., 1978; Stocks and Schumaker, 1979; Cotton and Hamkalo, 1981; Vassiler et al., 1981). A slow, reversible dissociation of DNA from core particles at room temperature was found by Yager and van Holde (1984), and the extent of dissociation was substantial when salt concentrations were abruptly increased from 0 to 0.5 M salt. Furthermore, Ausio et al. (1984a) demonstrated that dissociation increased as temperature was increased, at least up to 30 °C, the highest temperature they tried, and they also found that little dissociation occurred at salt concentrations less than 0.3 M (at 30 °C). At 0.6 M, Ausio et al. found about 20% of the DNA core particles dissociated. In comparing their results with those of Yager and van Holde, Ausio et al. concluded that the longer the core particle DNA, the more slowly the DNA dissociated when the salt concentration was increased. The present experiments on large fragments show that dissociation of DNA occurs from fragments containing H1 histone, as well as from core particles. It seems most likely that the free DNA observed in our experiments was, for the most part, present at the beginning of the programmed temperature rise of the scanning calorimetry. Our samples were exposed to their stated salt concentrations for approximately an hour at 20 °C for equilibration with the calorimeter before the scanning was started. Although the higher temperatures toward the end of the scanning period probably accelerated and shifted the equilibrium to some extent, the rate of dissociation must have been quite slow in order for the DNA transitions to be resolved from the denatured nucleoprotein transitions. The extent of DNA dissociation from large chromatin fragments can therefore be concluded to be comparable to that from core particles at room temperature.

The effect of fatty acid salts on the melting profiles is relevant to previous studies on major forces that stabilize the nucleosome. Eickbush and Moudrianakis (1978) argue for a dominant role for hydrophobic bonding in the first step of nucleosome formation and for hydrogen bonding in the second step where the histone octomer is completed. Ausio et al. (1984a,b) concluded that the primary force stabilizing the nucleosome could not be electrostatic. It was not obvious, therefore, how any amphipathic detergents would affect the nucleosome. The salts of fatty acids affected the melting profiles of chromatin the same way NaCl did, but in addition, the hydrophobicity of the fatty acid salts was a significant factor in destabilizing the nucleosome, at least at the elevated temperatures used in the present study. Regardless of the issue of dominance in the forces holding the nucleosome together, our results strongly suggest that both hydrophobic and electrostatic forces make substantial contributions to nucleosome stability. The contrast between the behavior of the nucleosome and that of denatured nucleoprotein underscores the importance of hydrophobic factors in the tertiary structure of the nucleosome.

Although the stability of the nucleosome was only modestly affected by pH in the physiological range, there was a substantial loss of stability at pH values less than 6.5. Perhaps this is related to the reversible conformational change of core particles observed near pH 7 by Libertini and Small (1984) using fluorescence anisotropy and circular dichroism; since this reversible conformational change was detected in 100 mM ionic strength, it might be physiologically relevant. Although it is risky to compare nucleosomes to their core histone octamers because the latter are quite unstable in physiological conditions, the conformational change seen near pH 7 (100 mM ionic strength) by Libertini and Small might correspond to the destabilization of histone octamers observed by Butler and Olins (1982) as the pH dropped below 5 in solutions of 5 mM ionic strength. There have been suggestions that this destabilization reflects critical hydrogen bonding through histidine or cysteine (Benedict et al., 1984).

Like those, who have worked with core particles, we have gained the impression that the nucleosome is a delicate structure, even when embedded in long nucleosomal strands. Its sensitivity to both electrostatic and hydrophobic factors provides potential for subtle regulation of its dynamics in transcription, replication, and repair as it interacts with specific domains of regulatory proteins and enzymes.

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