Histone Hyperacetylation: Its Effects on Nucleosome Conformation and Stability†

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ABSTRACT: We have prepared nucleosome particles from HeLa cells that have been subjected to butyrate treatment. Fractions containing different levels of acetylation have been obtained within the range 7–17 acetyl groups per nucleosome. We have put special emphasis in the characterization of the particles with the highest level of histone acetylation. At low to physiological ionic strengths, these nucleosomes exhibit only small differences in hydrodynamic behavior and circular dichroism from control particles with minimal acetylation. There are, however, significant differences in thermal denaturation and nuclease sensitivity. In terms of stability toward high salt, the hyperacetylated and control particles behave identically. A model that reconciles these results is proposed. The major conclusion from our results, however, is that, at physiological ionic strength and in the absence of factors other than acetylation, the highly hyperacetylated nucleosomes remain essentially folded.

Acetylation is one of the most widely studied posttranscriptional modifications of the histones. Since its discovery (Allfrey et al., 1964), it has been thought that this phenomenon might be associated with genetic biological activity, especially to the events involved in transcription. Early experimental evidence for such an association (Pogo et al., 1966) has been followed by a great number of reports supporting such an idea [see Doenecke & Gallwitz (1982) for a review]. Quite recently, using *Physarum polycephalum*, Waterborg and Matthews (1983) have been able to show a nice correlation between different degrees of histone acetylation and the different biological stages of the cell cycle and to distinguish specific patterns of S-phase and G-1-phase acetylation. Further indirect evidence comes from the fact that K562 human erythroleukemia cell variants that are resistant to growth inhibition by butyrate are also deficient in histone acetylation (Ohlson-Wilhelm et al., 1984).

In spite of all this evidence, there is not yet a complete agreement on the precise role of histone acetylation. Several groups have lately reported, in different organisms, an apparent lack of correlation between histone acetylation and transcriptional chromatin activity (Yukioka et al., 1983; Loidl et al., 1983). Indeed, it seems likely that acetylation may be involved in a number of processes. For example, histone hyperacetylation has recently been related to the events accompanying the displacement of the histones by protamines during spermiogenesis (Christensen et al., 1984).

At the molecular level, the association between histone acetylation and its biological role has been usually ascribed to a weakening of the histone–DNA interaction, as a consequence of the alteration in the charge balance introduced by such a chemical modification in the N-terminal regions of the histones. Again, controversy exists concerning the conformational consequences of such weakening, both at the nucleosome level and higher levels of chromatin organization. At the first level, a close similarity in the physical parameters for both acetylated and control nucleosome particles has been reported (Simpson, 1978). More recently, Bode et al. (1983) have suggested that nucleosome core particles undergo a dramatic change in conformation, in which the particle unfolds, upon reaching a "critical" level of histone acetylation. The apparent disagreement with previous reports could in principle be explained from the fact that no nucleosomal preparation, in the past, had reached that critical level of acetylation.

An unfolded nucleosome structure termed the "lexosome" has also been isolated recently from the transcribing rDNA chromatin in *Physarum* (Prior et al., 1983). The evidence for unfolding is based on three criteria: the much decreased sedimentation coefficient (~5 S),[1] reactivity of the H3 SH groups, and electron microscopic appearance. However, the relationship (if any) of these particles to the unfolded hyperacetylated nucleosomes postulated by Bode and co-workers is wholly obscure. The lexosome preparations contain relatively large amounts of non-histone proteins, and two such proteins have been postulated to be integral to the lexosome. No evidence concerning the acetylation state of these particles has been presented.

In this paper, we have taken advantage of and modified a fractionation method described by Perry and Chalkley (1981) to prepare nucleosome particles with different degrees of histone acetylation, both below and above the "critical" level postulated by Bode et al. (1983). This has allowed us to show that, independent of the acetylation degree and under physiological conditions, the structure and stability of the nucleosome particle remains essentially unchanged when compared to the control non-acetylated nucleosomes. This result is in agreement with earlier reports and does not support the unfolding model proposed by Bode et al. (1983).

Materials and Methods

HeLa cells (S3 strain), exponentially growing, were grown in suspension culture in minimum essential medium (Joklik modified, Gibco) supplemented with 10% fetal bovine serum, until a concentration of 5×10^5 cells/mL was achieved. At

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[1] Abbreviations: bp, base pair(s) (of DNA); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PIPES, 1,4-piperazinediethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; CD, circular dichroism; *Cfo*I, restriction endonuclease (type II); *Hin*fI, restriction endonuclease *Hin*fI; pBR322, plasmid pBR322; S, Svedberg unit (10^{-13} s).

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that time, in order to inhibit the internal deacetylases so as to increase the levels of histone acetylation (Riggs et al., 1977; Vidali et al., 1978), the cell cultures were brought to 10 mM sodium butyrate, 20–24 h before the beginning of the isolation of nuclei.

Preparation of Nuclei and Chromatin Digestion. A typical preparation was carried out on 6 L of cells that were harvested by low-speed centrifugation at 4 °C and then subjected to the treatment described by Perry and Chalkley (1981). The cell pellet was resuspended in 0.25 M sucrose, 60 mM KCl, 15 mM NaCl, 10 mM MES, 10 mM sodium butyrate, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton X-100, and 0.1 mM PMSF at pH 6.5 (buffer A) and centrifuged 10 min at 5000 rpm in a SS-34 Sorvall rotor at 4 °C. The pellet thus obtained was washed 2 more times following the same procedure. Then, the pellet was resuspended in 10 mM PIPES, 10 mM sodium butyrate, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 mM PMSF at pH 6.8 (buffer B) containing 50 mM NaCl and finally brought to an $A_{260} \simeq 40$ (about 2 mg of DNA/mL) with the same buffer.

The nuclei obtained in this way were incubated at 37 °C for 10 min and then digested for 3.5 min with micrococcal nuclease (Worthington) at 40 units/mL. The reaction was stopped by addition of EGTA up to a final concentration of 5 mM on ice.

Chromatin Fractionation and Nucleosome Isolation. The digested nuclei were centrifuged for 5 min at 9000 rpm in a SS-34 Sorvall rotor. This step yields two fractions: the supernatant, which comprises 8–9% of the starting chromatin and corresponds to the Mg^{2+}-soluble fraction of Perry and Chalkley (1981), and the pellet, which was subjected to a fractionation similar to that described by Sanders (1978). To this purpose, the pellet was suspended in buffer B containing in addition 100 mM NaCl and 2 mM EGTA and was extracted with this buffer for 20 min at 4 °C to solubilize a second fraction, comprising ~9% of the chromatin. This fraction exhibited a pattern of acetylation that was nearly identical with the one obtained in the previous fraction. The same operation was repeated, now with buffer B plus 200 mM NaCl and 2 mM EGTA. Finally, the remaining pellet was resuspended in 0.35 M NaCl, 10 mM Tris-HCl, pH 7.5, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM butyrate, and 0.2 mM PMSF (stripping buffer) and was extracted with this buffer. The amount of chromatin extracted in these two last steps accounts for 30–40% of the starting material. Alternatively, the pellet obtained after the first Mg^{2+} solubilization was, in some cases, resuspended and lysed in 0.25 mM EDTA and 10 mM sodium butyrate, pH 7.5.

All the fractions so obtained were concentrated to $A_{260} \simeq 20$ by using an Amicon cell, assembled with YM-100 membranes, and were subsequently dialyzed overnight against the above-mentioned stripping buffer. Histone H1 as well as virtually all of the non-histone proteins associated with the different fractions was then extracted in this buffer by gently shaking the samples for 3 h at room temperature in a way similar to that described by Ruiz-Carrillo et al. (1980), but in the presence of 1 g of AG50W-X2 (Bio-Rad) per milligram of DNA (as determined through the A_{260}). The stripped chromatin thus obtained was then concentrated in an Amicon cell until $A_{260} \simeq 20$ and was dialyzed overnight at 4 °C against 50 mM NaCl, 10 mM butyrate, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5, and 0.1 mM PMSF (buffer C).

Finally, the samples were redigested at 37 °C and at 20–25 units of micrococcal nuclease (Worthington)/mg of DNA for the times established through previous time-course analytical digestions carried out on aliquots of the same samples (Lutter, 1978). After the reaction was stopped by addition of EGTA to 5 mM, the samples so obtained were then loaded (with or without previous concentration) in 5–20% linear sucrose gradients in buffer C (without $CaCl_2$) and were centrifuged at 33 000 rpm for 16 h in a Beckman SW-40 rotor at 4 °C.

With this fractionation procedure, one can obtain nucleosome with different degrees of histone acetylation and completely free of non-histone contaminaton. We have called these fractions "a", "b", "c", and "d" in order of their decreasing level of acetylation. Nucleosome fraction a corresponds to the nucleosomes isolated from the first soluble chromatin fraction (i.e., that one which is soluble in buffer B containing 50 mM NaCl). b nucleosomes have been prepared from the chromatin fraction solubilized thereafter in buffer B containing 200 mM NaCl following the salt fractionation procedure described above. d nucleosomes correspond to those obtained by solubilization of the chromatin of the last pellet in stripping buffer. c nucleosomes are obtained from the chromatin fraction that is solubilized in 0.25 mM EDTA from the nuclear pellet immediately after micrococcal nuclease digestion.

In order to prepare non-acetylated control nucleosomes (fraction "e") from HeLa cells that had not been treated with butyrate, the nuclei were prepared and digested under the same conditions as described above, but in the absence of butyrate. After digestion, the nuclei were lysed in 0.25 mM EDTA, and the chromatin so obtained was further stripped and redigested under the same conditions used for the acetylated counterpart but always in the absence of butyrate.

^{32}P End Labeling and DNase I Digestion of Nucleosomes. Acetylated and control nucleosomes at $A_{260} \simeq 30$ were labeled at their 5′ ends with ^{32}P as described by Lutter (1979). After labeling, the samples were exhaustively dialyzed against 10 mM $MgCl_2$ and 25 mM Tris-HCl, pH 7.5, and were DNase I digested in this buffer at 40 DNase I units/mg of DNA at 37 °C. The reaction was terminated by addition of EDTA to 10 mM on ice, and immediately after that, the samples were brought to 0.05 mg/mL in proteinase K (Merck) and were incubated at 30 °C overnight. Then, SDS was added up to a final concentration of 0.3%, and the incubation was continued for 1 h at 37 °C. Finally, the samples were mixed with 1 volume of 60% formamide and 15% glycerol in TBE buffer (90 mM Tris–borate, pH 8.3, 2.5 M EDTA) containing 0.03% bromophenol blue and heated at 90–100 °C for 5 min and loaded on denaturing gels.

Gel Electrophoresis. Histones were analyzed either in SDS gels performed according to Laemmli (1970) or in 2.5 M urea–acetic acid gels with a slight modification of the method described by Hurley (1977). Solubilization of histones from chromatin, in this latter case, was routinely achieved by protamine displacement (Shaw et al., 1981) and was carried out as described by Reczek et al. (1982).

Two-dimension electrophoresis was performed by combining the above-mentioned methods with long urea–acetic acid slab gels (20 × 40 × 0.1 cm) for the first direction and 20 × 25 × 0.15 cm SDS slab gels for the second direction. For the quantification of the degree of histone acetylation, the relative amounts of the different modified forms for each histone were measured. For this purpose, the gels were photographed, and the negatives were scanned in a SL-504-XL Zeineh soft laser scanning densitometer (Biomed Instruments Inc., Fullerton, CA). The scans so obtained are shown in Figure 1 (panel III). Quantitative values were derived directly from these scans, under the assumption of a normal Gaussian distribution for each peak. On this basis and for a given histone, the area under each particular acetylation peak was determined. Fi-

nally, the acetylation level for this histone was calculated from

$$\sum_{n=0} n\Delta_n / \sum_{n=0} \Delta_n$$

where n is the number of acetyl groups assigned to a peak and Δ_n is the area under it.

DNA and native nucleosome particle gels were carried out as described elsewhere (Yager & van Holde, 1984). Single-stranded denaturing DNA gels (8 × 14 × 0.1 cm) in 7 M urea, for the analysis of the DNase I digestion products, were prepared according to a slight modification of the technique described by Lutter (1979). In our case, the matrix of the gel consisted of 12% polyacrylamide at an acrylamide to bis-(acrylamide) ratio of 10:1 (w/w).

Analytical Ultracentrifugation. Aliquots taken directly from the sucrose gradients used in the fractionation of nucleosomes were diluted in an excess of buffer (usually 5 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) containing different amounts of NaCl. The samples so prepared were run in a Beckman Model E analytical ultracentrifuge at 40000 rpm. The temperature was kept routinely at 20 °C through all the experiments performed. Scans were recorded by a photoelectric scanner and were analyzed as described by van Holde and Weischet (1978).

Circular Dichroism. Circular dichroism experiments were performed in a Jasco J41A spectropolarimeter. All the measurements were carried out on thermostated cells of 1-cm path length at 23 ± 1 °C. In all cases, the A_{260} of the sample was ≃0.8. CD spectra of nucleosomes with different levels of acetylation were recorded at a scale expansion of 0.5 mdeg/cm with a time constant of 1 s and a slit with of ~2 μm. In those experiments where the change of the maxima in the CD spectra (i.e., at 282.5 nm) was to be measured as a function of the salt concentration, the slit was kept completely open and the time constant was increased to 4–16 s. In such experiments, the salt concentration was increased by consecutive addition of known amounts of 4 M NaCl in 5 mM Tris-HCl (pH 8.0), which was slowly added under rapid stirring to the final NaCl concentration desired. The last addition consisted of solid NaCl so as to bring the nucleosome solution to 3 M NaCl. (The dilution factor of the sample in this last step was calculated by assuming a partial specific volume of 0.3 cm^3/g for the NaCl.) Since the time involved in this kind of experiment is quite long (close to 1 h), the base line for the buffer was recorded both at the beginning and at the end of the experiment. The base-line drift correction was then carried out through linear interpolation between the initial and the final values recorded.

Melting Profiles. These were carried out on a Cary 2200 spectrophotometer. The temperature was raised at constant rate of 1 °C every 4 min. Analysis of the data was performed as described by Li and Bonner (1971). The samples for these experiments were prepared by 1:10 (v/v) dilution in water of aliquots directly taken from the sucrose gradients used in the isolation of the nucleosomes. Therefore, the final composition of sample buffer was 5 mM NaCl, 2 mM Tris-HCl, pH 7.5, 1 mM butyrate, and ~1% sucrose. The starting A_{260} was set at 0.342 ± 0.001 for both the hyperacetylated and the control nucleosome particles.

Results

Fractionation and Characterization of Acetylated Nucleosomes. Perry and Chalkley (1981) have recently described a method to isolate acetylated chromatin by solubilization of the nuclear material, in the presence of $MgCl_2$, after a short digestion with micrococcal nuclease. As they have already shown, one can obtain by this method a mixture of mono- and oligonucleosomes with extremely high levels of histone acetylation. By combining this method with a fractionation procedure, similar to that described by Sanders (1978), we have found it pososible to prepare chromatin fractions with different degrees of histone acetylation. Further isolation and purification of nucleosomes from these samples is difficult to achieve, especially in the first soluble fractions, which contain the higher levels of histone acetylation. We find that these fractions contain, in addition to the histones, a considerable amount of non-histone material, which, in our hands, seems to be responsible for the ready aggregation and precipitation that occur upon concentration or extensive manipulation of such samples. Fortunately, under the specific conditions described under Materials and Methods for the stripping of histone H1, one can also coextract almost all of these non-histone protein components so as to allow further manipulation of the sample. By using this methodology, we have been able to prepare a broad spectra of nucleosome samples that exhibit different degrees of acetylation, as is shown in Figure 1. The level of acetylation for each of these samples is given in Table I. It is important to point out here the fact that, although some of the samples have a similar overall quantitative degree of acetylation, the distribution of acetylation levels between the various histones may vary considerably from one to another (see Figure 1 and Table I). We also show in Figure 1 the characteristics of the DNA from each fraction. As can be seen, the DNA length distribution and the average DNA length for each fraction is slightly different, presumably as a consequence of the difference in susceptibility during the trimming digestion. For the purpose of further comparison of these particles, it is important to mention here that is has already been shown (Ausio et al., 1984) that such small differences in DNA length do not have any major effect on the stability or the conformational parameters of nucleosome particles.

Table I: Acetyl Composition of Nucleosome Particles[a]

	nucleosome particle				
histone	a	b	c	d	e[b]
H2A	0.9	0.7	0.5	0.4	0.2
H2B	1.7	0.95	0.9	0.6	0.3
H3	2.7	1.2	1.35	1.1	0.6
H4	3.2	2.4	2.05	1.6	0.6
histone octamer[c]	17.0	10.5	9.6	7.4	3.4

[a] Values given for the individual histones are presented as moles of acetyl groups per mole of histone. Values for the octamer are moles of acetyl per mole of octamer. [b] Control particles, prepared from non-butyrate-treated HeLa cells. [c] This value corresponds to the sum of the degrees of acetylation of each histone multiplied by 2 (since two of each histone are present in the histone octamer).

Hyperacetylation Produces a Minor Conformational Change in Nucleosomes. Figure 2 shows the change in the sedimentation coefficients as a function of the ionic strength, for all the samples described in Figure 1, as compared to chicken nucleosomes (Yager & van Holde, 1984). Nucleosomes isolated from HeLa cells that had not been subjected to butyrate treatment (controls; sample e) show a behavior nearly identical with that observed for chicken core particles. On the other hand, acetylated nucleosomes exhibit a downward shift in sedimentation coefficient at all ionic strengths. Two important points arise from this graph. The first is that, in spite of the downward shift already mentioned, the change in conformation with increasing the salt, as measured from the drop in the sedimentation coefficient, is of the same magnitude and occurs in the same range of salt concentration for both the acetylated and the control nucleosomes. This is reflected

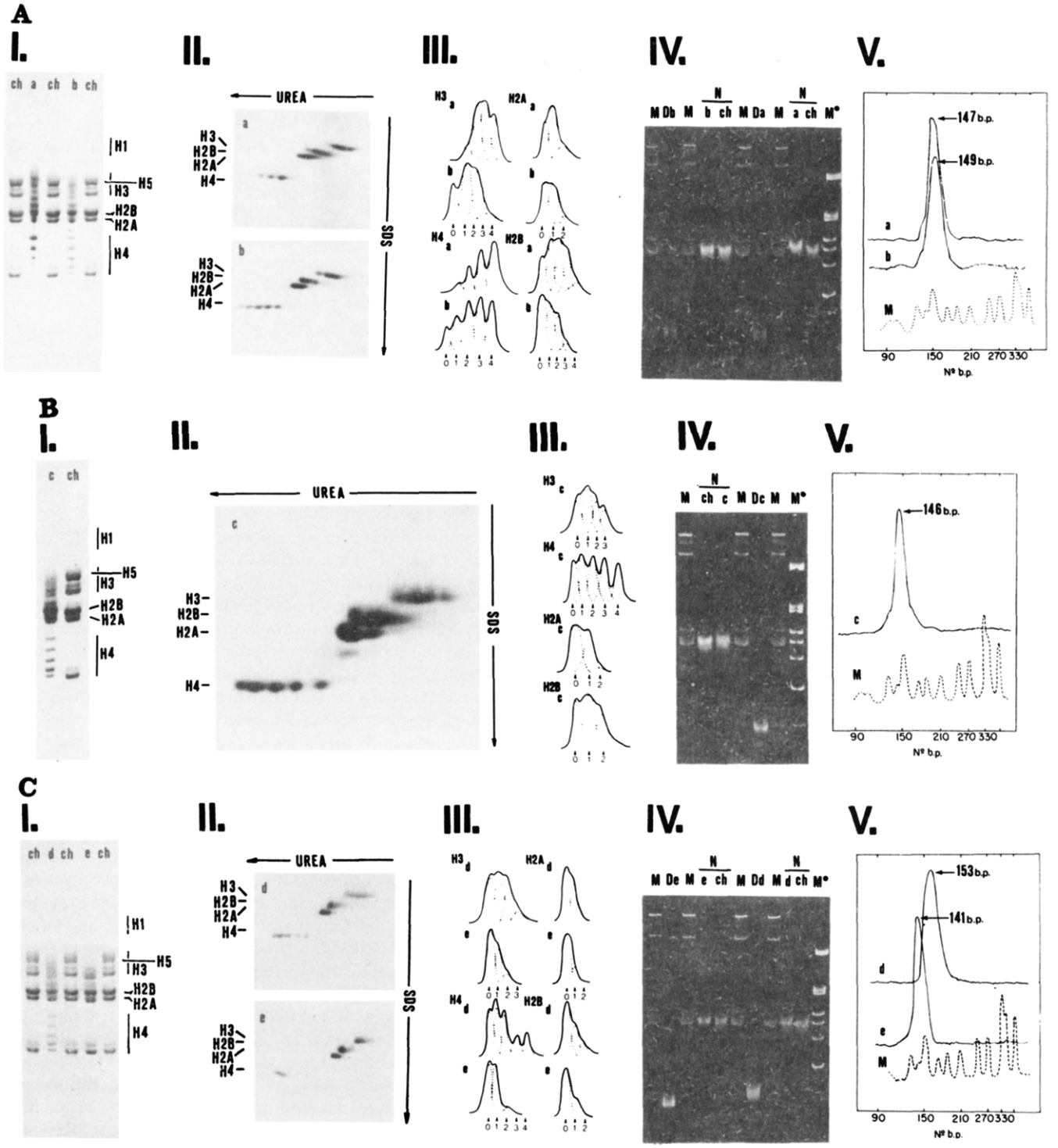

FIGURE 1: (A) Characterization of the nucleosome particles a and b, prepared as described under Materials and Methods: (I) acetic acid–urea gels of the histone contents; (II) double-dimension electrophoretic characterization of the histones; (III) scans obtained from (II) (the numbers indicate the number of acetyl groups); (IV) native DNA and nucleosome particle, 4% acrylamide gel; (V) scans obtained from (IV). The inset numbers indicate the number of base pairs (bp) corresponding to the peak maxima. Other symbols as follows: ch = chicken erythrocyte histones; Dx = DNA from particle x (where x = a, b, c, d, or e); N = nucleosome particles; M = DNA marker, pBR322 (*Cfo*I digested), M* = DNA marker, pBR322 (*Hinf*I digested); no. bp = number of base pairs. (B) Characterization of particle c, as in (A). (C) Characterization of particles d and e, as in (A).

in the parallel trend of the lines representing each set of data. The second point comes from the difficulty in distinguishing between acetylated particles with different degrees of acetylation. As can be seen in Figure 2, the sedimentation coefficient values for particles a–d do not decrease in any regular way with increasing level of acetylation; rather, the data scatter along the shadowed region in the figure. In other words, in the range of 7–17 acetyl groups per nucleosome, any trend in the sedimentation to diminish is within experimental error. Evidently, between a level of about three acetyl groups per nucleosome (as observed in the control particles) and about seven groups per nucleosome, a very small conformational change is triggered that decreases the sedimentation coefficient by about 10%.

This minor conformational change is also reflected in the circular dichroism of the nucleosomes. As can be seen from examination of Figure 3 [concentrating for the moment on the data of low ionic strength (50 mM)], the spectrum of the

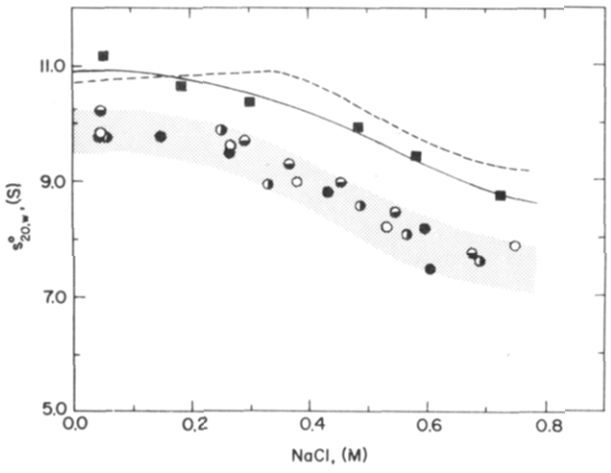

FIGURE 2: Sedimentation coefficients. $s_{20,w}$ of the different nucleosome particles, as a function of the ionic strength. (■) Control nucleosome particles e obtained from HeLa cells thaat have not been subjected to butyrate treatment; (◐) d particles; (◒) c particles; (○) b particles; (●) a particles. The dotted line (---) represents the data obtained from chicken erythrocyte nucleosome particles [from Yager & van Holde (1984)].

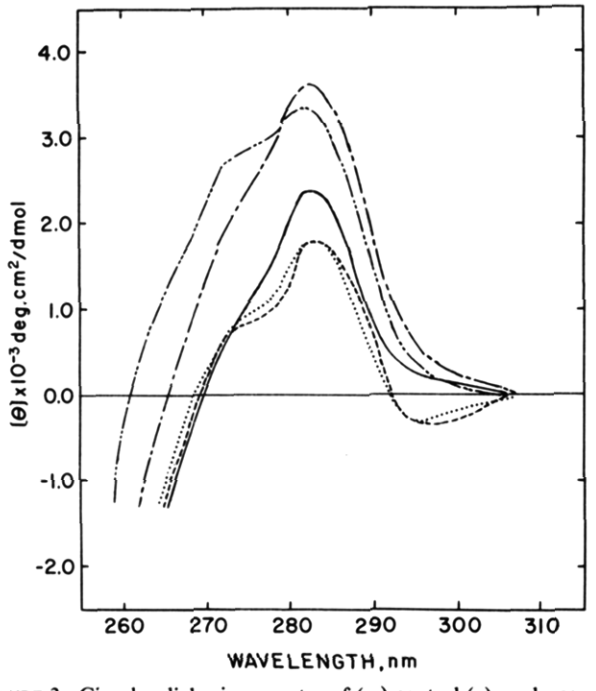

FIGURE 3: Circular dichroism spectra of (···) control (e) nucleosomes in 50 mM NaCl and 5 mM Tris-HCl, pH 8.0, (—) a nucleosomes under the same ionic conditions, (–·–·–) e nucleosomes in 0.6 M NaCl and 5 mM, Tris-HCl, pH 8, (– – –) a particles in 0.6 M NaCl and 5 mM Tris-HCl, pH 8, and (– · –) chicken erythrocyte mononucleosomes in 0.25 mM EDTA, pH 7.0 [from Cowman & Fasman (1978)].

highly acetylated a particles differs in two respects from that of the control particles: The small minimum at 295 nm has been lost, and the maximum at 282.5 nm has been slightly elevated. The change, however, is small when compared with that produced by high salt (see Figure 3 and below).

Neither the sedimentation coefficient change nor the alteration of the CD spectrum can correspond to any *major* unfolding, of the type postulated by Bode et al. (1983). Certainly, the degree of unfolding proposed for the lexosome is impossible to reconcile with these data. Yet, the change is real, and as Bode et al. suggested, occurs over a very narrow range of acetylation values. Since the N-terminal tail of each nucleosome contains about 10 basic groups, this change occurs

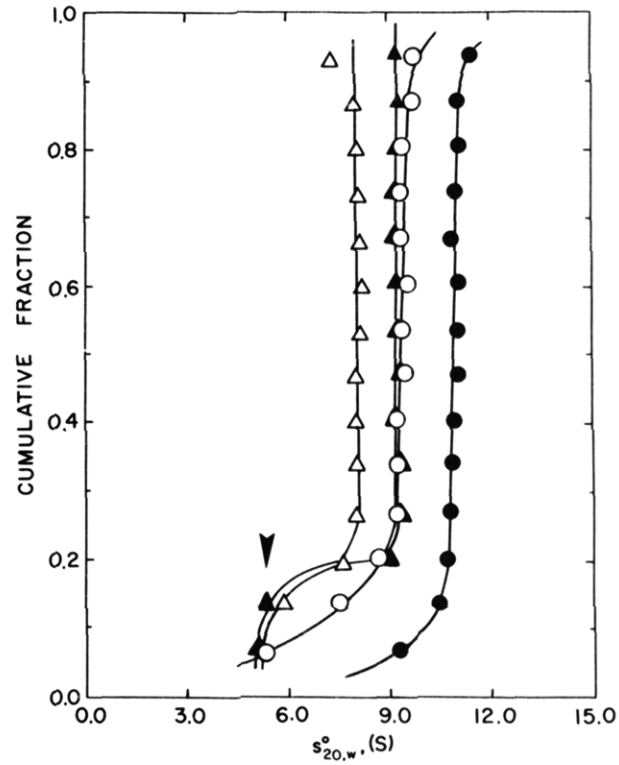

FIGURE 4: Integral distributions of the sedimentation coefficients determined according to the method of van Holde and Weischet (1978): (●) e control nucleosome particles in 50 mM NaCl, 5 mM Tris-HCl, and 0.1 mM EDTA, pH 8.0; (○) a nucleosome particles under the same conditions; (▲) e nucleosomes at 0.6 M NaCl, 5 mM Tris-HCl, and 0.1 mM EDTA, pH 8.0; (△) a particles under the same buffer conditions as before. The arrow indicates the sedimentation coefficient corresponding to free nucleosomal DNA, which appears as a consequence of partial dissociation of the nucleosomes upon increasing the NaCl concentration (Ausio et al., 1984; Yager and van Holde, 1984).

when only about 10% of them have been neutralized. Further acetylation (up to about 20% of the positively charged tail residues) appears to have no further effect on the hydrodynamic behavior of the particles. It should be emphasized that in all of our studies the control particles were prepared in exactly the same fashion as the particles from butyrate-treated cells.

Hyperacetylation Has No Effect on the Stability of Nucleosomes in High Salt. Since acetylation of histones reduces the number of possible charge interactions between histone tails and the DNA, one might expect that it would increase the tendency of nucleosomes to dissociate and/or undergo conformational changes in high salt. Surprisingly, this is not the case. We find that the stability of the nucleosome particles as a function of the environmental ionic strength is the same, both for the control and for the particles with different levels of acetylation. In the first place, the dissociation of DNA from nucleosomes as a consequence of the increase in the salt within the range 0.1–0.7 M NaCl does not depend on the degree of histone acetylation. This is demonstrated in Figure 4 and in other data not shown. Both the highly acetylated a particles and the controls exhibit about 20% dissociation in 0.6 M NaCl, a result very similar to that found for chicken erythrocyte nucleosomes under these conditions (Ausio et al., 1984; Yager & van Holde, 1984).

In addition to partial dissociation, nucleosomes respond to increased ionic strength by a reversible conformational change. One aspect of this change is illustrated by the decrease in sedimentation coefficient with increasing NaCl concentration

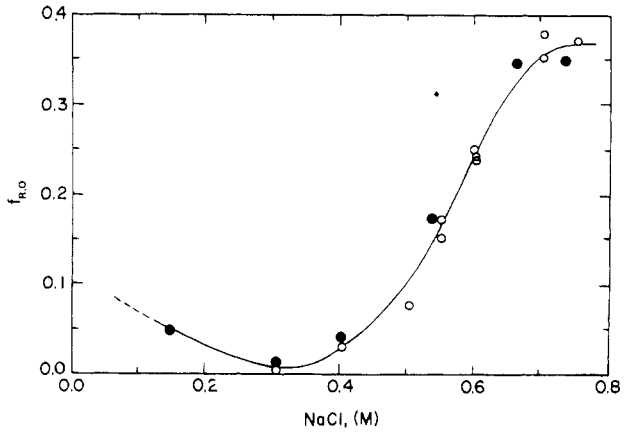

FIGURE 5: Salt-induced increase in circular dichroism of nucleosome core particles as determined at the maximum (282.5 nm) of their CD spectra. $f_{R,O}$ represents the fraction of DNA becoming freed of the nucleosomal constraints introduced by the N-terminal regions (tails) of the histones. (Closed circles) a nucleosome particles; (open circles) chicken erythrocyte nucleosome particles (Yager et al., submitted for publication).

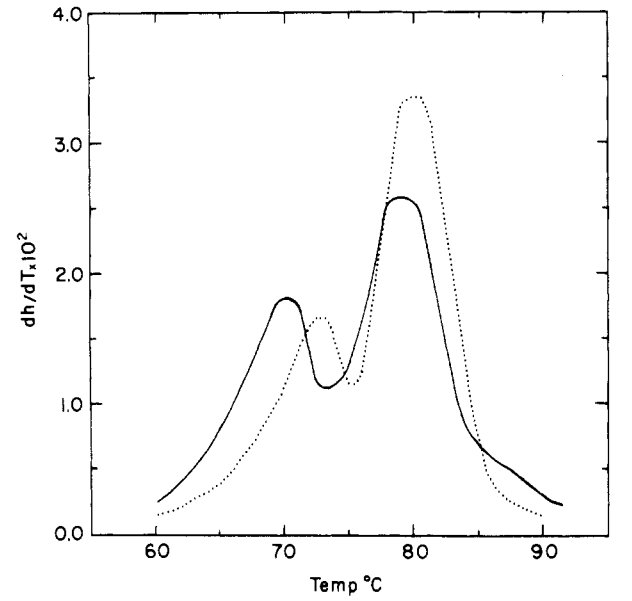

FIGURE 6: Thermal denaturation profiles of (···) control and (—) highly hyperacetylated a nucleosome particles.

shown in Figure 2. As pointed out above, this change takes place over the same salt concentration range with control and hyperacetylated nucleosomes. Another feature of this conformational change is a substantial increase in the circular dichroism of nucleosomes above 0.3 M salt (Yager et al., submitted for publication). This is illustrated in Figures 3 and 5. The major point to be made here is that this change is virtually identical, both in its magnitude and salt dependence, for highly acetylated HeLa nucleosomes and chicken erythrocyte nucleosomes (Figure 5). Although the molecular details of the reversible, salt-induced conformational change are still unknown, it is clear from the above data that the sites of hyperacetylation cannot be involved in those DNA–histone contacts that are modified in this process.

Hyperacetylated Nucleosomes Exhibit Modified Thermal Denaturation and DNase I Susceptibility. All the evidence presented above clearly indicates that nuclesome particles with or without acetylation behave very similarly with respect to stability. Nevertheless, they exhibit some differences that reflect their compositional diversity. Examples of such differences are found in the melting behavior and the DNase I

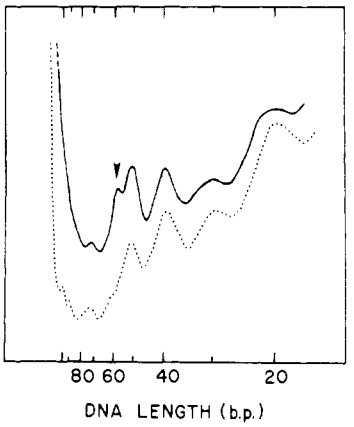

FIGURE 7: Scan of a denaturing gel electrophoresis of products of DNase I digestion of ^{32}1P 5′ and labeled nucleosomes: (···) control nucleosomes; (—) a nucleosomes. The arrow indicates the position of the site located 60 nucleotides away from the 5′ end. The times of digestion were 2 min for the acetylated particles and 8 min for the control nucleosomes.

digestion of such particles. The results obtained in the first case are shown in Figure 6. As can be seen there, the first transition of the typical biphasic melting profile expected for nucleosomes occurs at lower temperature (70 °C) for the acetylated particles, when compared to the control nucleosomes (73 °C). Moreover, this first transition accounts for approximately 46% of the total hyperchromicity in the case of the acetylated particles, whereas in the control nucleosomes this transition corresponds only to about 27%. The first observation would reflect the fact that part of the nucleosomal DNA, in the acetylated particles, has a structure more similar to that of free DNA in solution, most probably as a consequence of a certain degree of relaxation achieved upon release of the acetylated terminal regions of the histones. Furthermore, the amount of DNA maximally stabilized by protein–DNA interactions has become substantially decreased upon acetylation, as reflected by the quantitative increase in the first melting transition. Once again, this indicates a strong concomitance between tail release and the premelting transition of the core particles, as already pointed out by Walker (1984). These observations are also in good agreement with those of Bode et al. (1980).

The first transition in the thermal denaturation profile has been ascribed to the melting of DNA 25–30 bp from each end of the nucleosomal coil (Weischet et al., 1978; Simpson, 1979). The fact that the *amount* of DNA melting in this first step is markedly increased by hyperacetylation suggests that an effect of such modification is to weaken DNA–histone contacts further into the DNA coil. Support for this concept comes from DNase I digestion studies. In the first place, we find, in agreement with Simpson (1978), that the hyperacetylated particles are more rapidly digested than the control particles. More specifically, we also find (Figure 7) that a site located ~60 bp from the ends of the DNA shows greatly increased susceptibility.

Discussion

In this paper, we report a method for the preparation of substantial quantities of nucleosomes with very high levels of histone acetylation. The number of acetyl groups per nucleosome in our a fraction is considerably greater than in the particles studied by Simpson (1978), or in the earlier experiments of Bode et al. (1980). In later experiments, Bode et al. (1983) were able to obtain one fraction comparable to our a fraction, but only in smaller quantities through preparative

gel electrophoresis. The highest acetylation values we obtain approach the maximum possible through in vivo modification. If in vivo acetylation has significant effects on nucleosome conformation and/or stability, we should observe them with such samples.

The results are clear. Hyperacetylation does have a very small effect on the properties of nucleosomes at low to physiological ionic strength. The sedimentation coefficient is decreased by about 10%, and there is a corresponding small increase in the molar ellipticity in the DNA bands. Neither could conceivably correspond to the kind of major unfolding postulated by Bode et al. (1983). It should be noted that the conclusions of Bode et al. were based mainly on changes in electrophoretic mobility on gels. Sedimentation provides a much more direct measure of changes in hydrodynamic properties.

Such a small change in the frictional properties of the core particles could result from a partial release of histone tails as a consequence of their acetylation. The fact that H3 and H4 exhibit the maximum modification in our preparations suggests that the tails of these two histones may be specifically involved in the small decrease in S observed for the hyperacetylated particles at low ionic strength. Such a postulate is supported by the observation by Whitlock et al. (1983) that acetylation results in increased accessibility of the N-terminal region of H3 to phosphorylation by a Ca^{2+}-dependent kinase. Immunological studies of the effect of histone H4 acetylation by Muller et al. (1982) revealed decreases in reactivity toward antibodies to H3 and H2A. The most dramatic effects were observed with H3 reactivity, again suggesting conformational changes in the H3–H4 regions of the histone core.

It is also significant that the control HeLa nucleosomes and fraction a are both homogeneous in sedimentation (Figure 4). This would seem to rule out the possibility that the effect of butyrate treatment on nucleosome properties results from the decrease in H2A phosphorylation observed by Boffa et al. (1981). Since Dolby et al. (1979) have shown that a fraction of HeLa H2A is phosphorylated throughout the cell cycle, one would expect to see heterogeneity in the control particles, were their sedimentation behavior sensitive to H2A phosphorylation.

Insofar as stability toward salt-induced dissociation and conformational change is concerned, hyperacetylation appears to have no effect at all. The simplest interpretation of this somewhat surprising result is that entirely different and independent regions of DNA–histone interaction are involved in the small acetylation-induced changes and the much more pronounced salt-induced changes. Some aid in a tentative assignment of these regions is provided by the results of thermal denaturation and DNase I digestion experiments. Both suggest that the primary effect of hyperacetylation is to weaken DNA–histone interactions in the inner 80–100 bp of the DNA coil. This is readily understandable, for histones H3 and H4, which are the primary targets of hyperacetylation, make their major contacts with the DNA in this region (Richmond et al., 1984). If the "tails" of these histones are hyperacetylated, either partial tail release or a general lossening of the structure could account for the small changes seen in hydrodynamic and optical properties. Furthermore, this should allow increased DNase I susceptibility in the regions ~60 bp from the ends and permit the first stage in thermal denaturation to make a greater incursion into the nucleosomal DNA.

The implication, then, is that the *salt-induced* conformational change involves primarily the interaction of H2A and H2B tails with the *end* regions of the nucleosome core. This is consistent with the observation that the amount of DNA involved (about 54 bp from Figure 5) corresponds approximately to the amount participating in the first stage of thermal denaturation in control nucleosomes, which is known to be end DNA. That this region should not participate in the acetylation-induced transition is consistent with the fact that H2A and H2B are lightly acetylated even in the hyperacetylated particles.

This transition must involve histone tail release, as indicated by the NMR data of Walker (1984). It must also involve a relaxation rather than a release of the DNA in the nucleosome, for Greulich et al. (1985) have found from low-angle X-ray scattering that this transition does not involve a significant increase in the DNA-dominated radius of gyration. This result has been confirmed by neutron scattering by Yager et al. (submitted for publication). Such a model makes the specific suggestion that one role of histone hyperacetylation may be to prepare the *inner* DNA coil on the nucleosome for displacement from the histone core, either in transcription or in protamine displacement. If such were the case, hyperacetylation would be a necessary but not sufficient condition for such processes.

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Effects of Denaturants on Amide Proton Exchange Rates: A Test for Structure in Protein Fragments and Folding Intermediates†

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ABSTRACT: A method for detecting structure in marginally stable forms of a protein is described. The principle is to measure amide proton exchange rates in the absence and presence of varying concentrations of a denaturant. Unfolding of structure by the denaturant is reflected by an acceleration of amide proton exchange rates, after correction for the effects of the denaturant on the intrinsic rate of exchange. This exchange-rate test for structure makes no assumptions about the rate of exchange in the unfolded state. The effects of 0–8 M urea and 0–6 M guanidinium chloride (GdmCl) on acid- and base-catalyzed exchange from model compounds have been calibrated. GdmCl does not appear to be well-suited for use in the exchange-rate test; model compound studies show that the effects of GdmCl on intrinsic exchange rates are complicated. In contrast, the effects of urea are a more uniform function of denaturant concentration. Urea increases acid-catalyzed, and decreases base-catalyzed, exchange rates in model compounds. The exchange-rate test is used here to study structure formation in the S-protein (residues 21–124 of ribonuclease A). In conditions where an equilibrium folding intermediate of S-protein (I_3) is known to be populated (pH 1.7, 0 °C), the exchange-rate test for structure is positive. At higher temperatures (>32 °C) I_3 is unfolded, but circular dichroism data suggest that residual structure remains [Labhardt, A. M. (1982) *J. Mol. Biol. 157*, 357–371]. Under these conditions (pH 1.7, 45 °C) the exchange-rate test is negative, indicating that any residual structure in thermally unfolded S-protein does not have a detectable effect on amide proton exchange rates.

In native proteins, amide proton (peptide NH) exchange rates are often retarded by a factor of 10^6 or greater, compared to exchange rates from unfolded states. The large reduction in exchange rates is thought to be caused chiefly by hydrogen bonding (H-bonding) and solvent exclusion (Barksdale & Rosenberg, 1982; Englander & Kallenbach, 1983; Richards, 1979; Wagner & Wüthrich, 1982; Woodward et al., 1982). Amide protons are H-bond donors in α-helices and β-sheets; there is also a large reduction in solvent-accessible surface area when these structures are formed [reviewed by Richards (1977)].

Factors other than structure can also have significant effects on the rates of amide proton exchange. In unstructured model compounds, both nearest-neighbor inductive effects (Molday et al., 1972) and charge effects [Kakuda et al. (1971); Kim & Baldwin (1982); see also Matthew & Richards (1983)] are known to influence the intrinsic rate of amide proton exchange. This has made it difficult to correlate observed exchange rates with the presence of structure, especially when stability of the structure is low (i.e., the extent of protection from exchange is less than 100-fold).

Here we describe a method to detect relatively unstable elements of structure. We use denaturants to unfold the polypeptide; exchange from amide protons involved in structure is accelerated by unfolding. A principal advantage of this structure test is that no assumptions are made about the exchange rate constants in the unfolded state. It is necessary that the structure retards amide proton exchange rates and that the structure is unfolded by denaturant.

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