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Unfolding of 175-Base-Pair Nucleosomes†

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ABSTRACT: Calf thymus nucleosomes containing 175 base pairs of DNA unfold in two steps as the salt concentration is lowered, as detected by electric dichroism measurements. The transition midpoints are at 2.9 and 1.1 mM ionic strength at 7 °C with at most a small dependence on temperature. We identify the product of the 2.9 mM transition as an expanded disklike structure similar to the product of the 1.0-1.3 mM unfolding transition of 146-base-pair nucleosomes. The product of the 1.1 mM transition in 175-base-pair nucleosomes is elongated into a more asymmetric particle.

The structure of transcriptionally active chromatin is a subject of intense current investigation, whose general objective is to clarify how DNA which normally is bound to histones becomes accessible to RNA polymerase and regulatory proteins in general. Recent evidence has shown that active genes are highly susceptible to DNase I digestion (Gottesfeld et al., 1975; Weintraub & Groudine, 1976; Garel & Axel, 1976; Weisbrod et al., 1980; Giri & Gorovsky, 1980) although it is not yet certain whether the primary sensitive sites are intra- or internucleosomal. Weintraub & Groudine (1976) and Groudine et al. (1978) have observed that in globin and integrated viral genes the enhanced nuclease sensitivity could be maintained in isolated core nucleosomes, possibly implying a different conformation for active and inactive nucleosomes. Along these lines, Giri & Gorovsky (1980) concluded that the DNase I sensitivity of activated ribosomal genes arises from a reversible alteration of the core structure during activation. On the other hand, Garel & Axel (1976) did not find retention of DNase I sensitivity in isolated nucleosomes from active ovalbumin genes.

One way to characterize and compare the ability of nucleosomes to unfold is to examine their response to perturbing agents, of which low salt concentration is the most studied. Reduced ionic strength causes a reversible unfolding of core particles without loss of proteins; the transition has been characterized by hydrodynamic (Gordon et al., 1978; Harrington, 1981), electron microscopic (Oudet et al., 1977), electrooptical (Wu et al., 1979), and fluorescence (Dieterich et al., 1977, 1979; Dieterich & Cantor, 1981) techniques. While there has been some variability in the results, the emerging consensus is that there is a single unfolding transition, whose mechanism is complex, centered at about 1 mM mo-

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novalent salt concentration. The low salt form is somewhat expanded compared to the compact structure, as revealed by its increased translational and rotational frictional coefficients. Presumably the net negative charge of the core particle leads to repulsive electrostatic forces which are sufficient to disrupt the compact structure when screening counterions are removed.

Nucleosomes containing more than 146 base pair(s) (bp) of DNA may differ from core particles in their salt stability for at least two reasons. The extra DNA, with its additional negative charge, might be expected to be destabilizing. In addition, the cross-linking studies of Belyavsky et al. (1980) show significant differences in the attachment of histone proteins to DNA in 175-bp nucleosomes compared to core particles. Recognizing that larger nucleosomes are probably closer than core particles to the structure found in native chromatin, we have characterized the salt unfolding pattern of 175-bp calf thymus nucleosomes depleted of histone H1, using hydrodynamic and linear dichroism measurements. The results show that a second and more extensive unfolding step is found for these nucleosomes, compared to core particles. Thus, for the 175-bp nucleosomes, we propose three salt-dependent states: *compact* (native), *expanded* (a disklike structure of increased diameter comparable to the unfolded state of core particles), and *extended* (in which the particle is elongated along the DNA superhelix axis). Dichroism measurements reveal the two-step nature of the unfolding process, since the dichroism amplitude first decreases and then increases. If one allows for the probable inability of hydrodynamic methods to resolve the two transition steps, our observations are in general agreement with those reported by Burch & Martinson (1980) for nucleosomes containing large DNA.

Materials and Methods

Nucleosome Preparation. Nucleosome samples of 175 bp were prepared as previously reported (Crothers et al., 1978). The fractions from a Bio-Gel A-5m column were pooled and further purified by treatment with 100 mM KCl to precipitate the last trace of H1-containing particles. The supernatant was then loaded on a linear 5–20% sucrose gradient in TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5). The gradient was run for 24 h in an SW 27 rotor at 25 000 rpm. Fractions were assayed for nucleosomal particles, free DNA, DNA length, and histone proteins by gel electrophoresis. Figure 1 shows the results of a typical preparation. The purity of the particles obtained was monitored by the electrophoretic pattern of the intact particle, shown in the two right-hand lanes in Figure 1. The contamination of 175-bp nucleosomes by 146-bp particles evident in the gel electrophoresis patterns of our earlier preparation (Crothers et al., 1978) was removed by the sucrose gradient step employed here. Material prepared in this way showed little overlap between the gel bands of 146- and 175-bp nucleosomes. The DNA bands, in contrast, overlap appreciably (second and third lanes from the left in Figure 1), especially when heavily loaded as in Figure 1. This contrast suggests that the two nucleosomal particles may differ in some characteristic such as conformation, in addition to the average size of DNA present. A sample of core particles of the kind used for crystallization studies was kindly provided by Dr. Aaron Klug, Medical Research Council (MRC), Cambridge.

Electric Dichroism. Dichorism measurements were performed as described earlier (Hogan et al., 1978). The reduced dichroism ρ is defined by

$$\rho = \frac{A_\parallel - A_\perp}{A} = \frac{3}{2}\left(\frac{A_\parallel - A}{A}\right) \qquad (1)$$

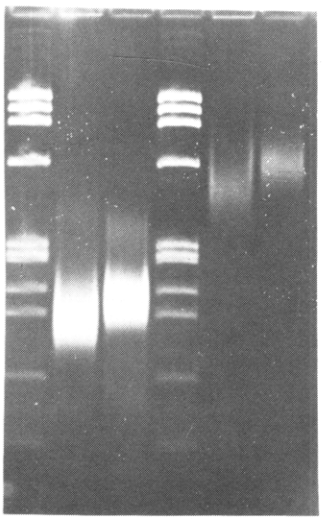

FIGURE 1: Polyacrylamide gel electrophoresis (5%) of nucleosomes and DNA. From left to right, *Hae*III restriction digest of $\phi X174$ DNA, DNA from 146-bp nucleosome, DNA from 175-bp nucleosome, restriction digest, 146-bp particle, and 175-bp particle. Incomplete protein removal accounts for the low mobility of the nucleosomal DNA bands.

where $A_\parallel$ and $A_\perp$ are the absorbances when the incident light is plane polarized parallel and perpendicular respectively, to the electric field axis and A is the absorbance in the absence of the field. We recently showed that the dichroism of superhelixes oriented perpendicular to the field axis is a function of the number of turns of DNA it contains. Specifically (and correcting an earlier error[1])

$$\rho_\perp = -\frac{3}{8}\left(\frac{\langle 3\cos^2\alpha - 1\rangle}{N + 1 - \delta/\pi}\right)[(N + 1 - \delta/\pi) \times (3\cos^2\beta - 1) - [3B(\pi - \delta)/\pi]\sin^2\beta] \qquad (2)$$

where N is the number of complete DNA turns, $N + (\pi - \delta)/\pi$ is the total number of turns, α is the angle of the DNA transition moments relative to the double-helix axis, β is the superhelix pitch angle, and $B = [\sin(2\delta)]/[2(\pi - \delta)]$. If the superhelix orients with its axis parallel to the field, then the dichroism ρ is (Crothers et al., 1978)

$$\rho = \tfrac{3}{4}(3\cos^2\beta - 1)(\langle 3\cos^2\alpha - 1\rangle) \qquad (3)$$

The observed orientation time, $\tau^{(r)}$, in the field was related to the rotational diffusion constant as described previously (Crothers et al., 1978).

Variation of the ionic strength was achieved by dilution of TE buffer. Ionic strengths were calculated by assuming full protonation of Tris and double ionization of EDTA at pH 7.5. Measured dichroism values were extrapolated to infinite field by using the equations for a field-independent dipole moment.

Dynamic light scattering measurements utilized a Malvern correlator described previously (Mandelkern et al., 1981). Typical nucleosome concentrations used were 100–300 μg/mL; no dependence of correlation time, $\tau^{(t)}$, on concentration could be detected. Decay curves were independent of truncation of the number of chanels and were fitted within experimental scatter by single exponentials.

[1] This equation was given incorrectly by Crothers et al. (1978), although the correct equation was used for the figures and all calculations in that paper. We thank Dr. C. Houssier for pointing out this mistake to us.

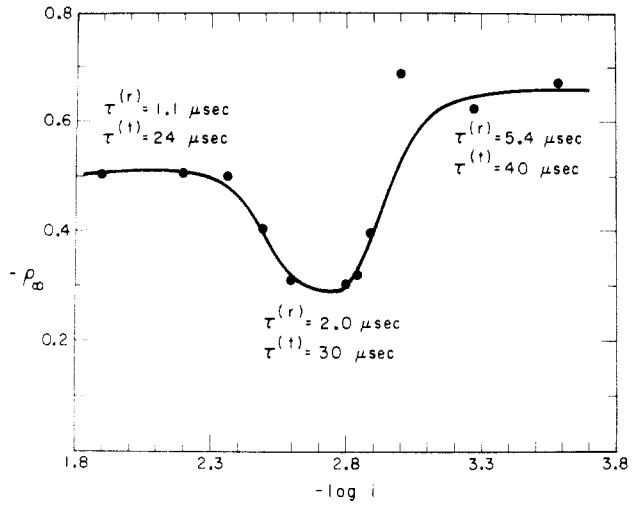

FIGURE 2: Low salt induced transition curve for 175-bp nucleosome. Reduced dichroism at perfect orientation (vide text) has been plotted against log (ionic strength); temperature 7 °C. Different ionic strengths are reached by the combination of dialysis and dilution. Rotational ($\tau^{(r)}$) and translational ($\tau^{(t)}$) correlation times for nucleosomes in the three states are given.

Results

Confirmation of a Single Transition for Core Particles. We investigated a sample of core particles of the type used for crystallographic studies, prepared at the MRC laboratory (Cambridge) and kindly provided by Dr. Aaron Klug. The dichroism variation was identical within experimental error with the single transition we reported earlier for core particles (Wu et al., 1979), with a transition midpoint of ~1.2 mM at T = 7 °C.

Two Transitions for 175-bp Nucleosomes. Figure 2 shows the extrapolated dichroism variation found when ionic strength is varied. In contrast to the results with core particles, which show a single sigmoid transition, we find first a decrease and then an increase in the (absolute) reduced dichroism amplitude. The midpoints of the two transition steps are (T = 7 °C) about 2.9 and 1.1 mM. Techniques which do not resolve the two transitions should detect a single (wider) transition at the geometric mean of the two concentrations, or about 1.8 mM, assuming a logarithmic ion concentration plot is used to evaluate the data. The remainder of our experiments are designed to characterize the two unfolded states observed. No detectable (±0.2 mM) dependence of this transition curve on temperature was found between 7 and 20 °C.

Ionic Strength Dependence of the Apparent Dipole Moment. Core particles have a field-independent dipole moment of about μ = 1200 D (Crothers et al., 1978), independent of the ionic strength as long as the structure is not altered. A similar behavior, with μ = 2600 D, is found for the expanded form of core particles. On the other hand, the apparent saturating dipole moment of DNA (Hogan et al., 1978), and of core particles extensively unfolded by ethidium binding (Wu et al., 1980), is strongly dependent on ionic strength. We associate this property with induction of a dipole moment by polarization of the ionic atmosphere; usually this behavior is characteristic of elongated charged particles.

We found the dipole moment of compact 175-bp nucleosomes to be constant at μ = 1190 ± 100 D over the range of 15–5 mM ionic strength where no change in dichroism is detected. For the intermediate unfolded state, only a narrow range of salt concentrations was accessible (2.6–1.5 mM), but we found a constant value of μ = 1960 ± 200 D, close to the value found for the unfolded form of core particles. Figure

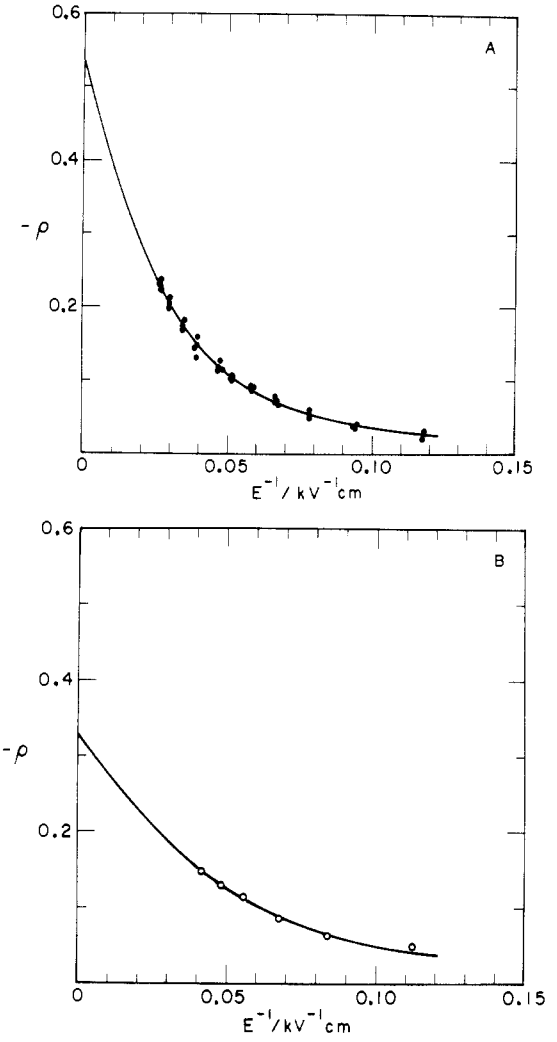

FIGURE 3: (A) Field dependence of reduced dichroism of the compact 175-bp nucleosome. Different points at the same field indicate measurement at ionic strengths between 13.0 and 4.33 mM; temperature 7 °C. (B) Field dependence of reduced dichroism of the intermediate (expanded) form of 175-bp nucleosome at 2 mM; temperature 7 °C. Solid lines are theoretical field dependence curves calculated for best fit to the data.

3 shows typical field extrapolation plots for the compact (Figure 3A) and intermediate (Figure 3B) unfolded states.

Below 1 mM ionic strength, a strong variation of dipole moment with ionic strength was observed, as illustrated by the field extrapolation curves in Figure 4. As shown in Figure 5, the apparent dipole moment (at saturating fields) is linearly dependent on the reciprocal square root of the ionic strength, as we found earlier for DNA (Hogan et al., 1978) and other systems (Wu et al., 1980).

Rotational Diffusion Coefficients. We measured the field-induced orientational rise times for compact 175-bp nucleosomes and the two unfolded forms. As previously described (Crothers et al., 1978), dextran T500 was used to enhance the viscosity of the medium, with extrapolation to zero dextran concentration to determine the rotational relaxation time in aqueous solution. For the compact and intermediate states, in which μ is independent of salt concentration, we determined the rotational diffusion constant $D^{(r)}$ from $D^{(r)} = 1/[2\tau^{(r)}]$, because the presence of a permanent moment allows the molecule to orient in only one direction in the field. For the final unfolded state, we took, by analogy with DNA (Hogan et al., 1978) and other unfolded states of nucleosomes (Wu et al., 1980), $D^{(r)} = 1/[6\tau^{(r)}]$. In this case, polarization of the ion atmosphere is equally likely in either direction along

Table I: Properties of 175-bp Nucleosomes in Different States

form	ρ_∞ [a]	μ_{app}/D [a]	$\tau^{(r)}$ (μs) [b]	$D_{20°C}^{(r)} \times 10^{-5}$ (s^{-1})	$\tau^{(t)}$ (μs) [c]	$D_{20°C}^{(t)} \times 10^{7}$ (cm^2 s^{-1})
compact (above 5 mM)	−0.49	1190 ± 100	0.7	6.8	24 ± 2	3.55
expanded (2.6-1.5 mM)	−0.33	1960 ± 200	1.3	3.7	30 ± 2	2.85
elongated (below 1 mM)	−0.65	variable with ionic strength (Figure 5)	3.6	0.46	44 ± 4	1.94

[a] Measured at 7 °C by using equations for a field-independent dipole. [b] Field-induced orientation time, measured at 7 °C and corrected to 20 °C by the ratio $[\eta_{T_2}(H_2O)/\eta_{T_1}(H_2O)]$ (T_1/T_2). [c] Measured at 20 °C, at scattering angle $\theta = 90°$.

Table II: Nucleosome Stability

sample	transition midpoint ionic strength (mM)	method	ref
core particle [a]	1 (1), 7.5 (2)	sedimentation, diffusion	*b*
core particle (reconstituted)	1	fluorescence of labeled protein	*c*
core particle	1.2, (T = 7 °C)	dichroism, rotational relaxation	*d*
core particle	3	fluorescence of labeled protein	*e*
173- and 250-bp nucleosome (reconstituted)	7	fluorescence of labeled protein	*e*
core particle	0.2	sedimentation, tyrosine fluorescence	*f*
core particle	1.2	sedimentation	*g*
169- and 203-bp nucleosome	1.6	sedimentation	*g*
core particle	1.1	fluorescence	*h*
core particle [a]	0.6 (1), 7 (2)	flow birefringence, intrinsic viscosity	*i*
175-bp nucleosomes	1.1 (1), 2.9 (2), (T = 7 °C) (mean = 1.8 mM)	dichroism	*j*
core particle (MRC sample)	1.2 (T = 7 °C)	dichroism	*j*

[a] These samples contain appreciable amounts of larger DNA, and had been frozen. [i] [b] Gordon et al. (1978). [c] Dieterich et al. (1979). [d] Wu et al. (1979). [e] Dieterich et al. (1980). [f] Libertini & Small (1980). [g] Burch & Martinson (1980). [h] Dieterich & Cantor (1981). [i] Harrington (1981). [j] This study. (1) and (2) indicate transition number.

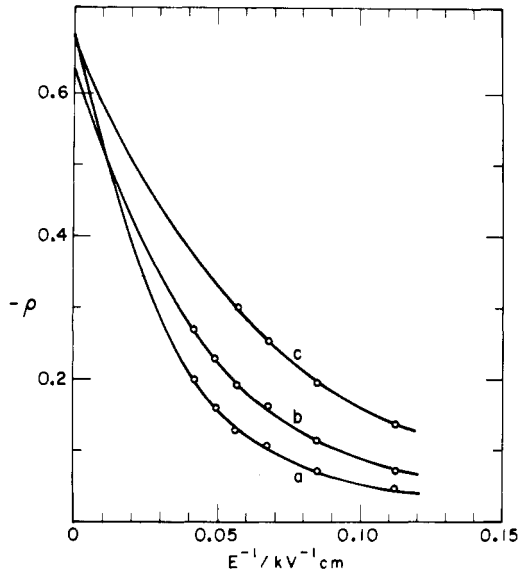

FIGURE 4: Field dependence of the reduced dichroism of the extended form. (a), (b), and (c) are measured at ionic strengths of 1.08, 0.54, and 0.26 mM, respectively. Temperature 7 °C.

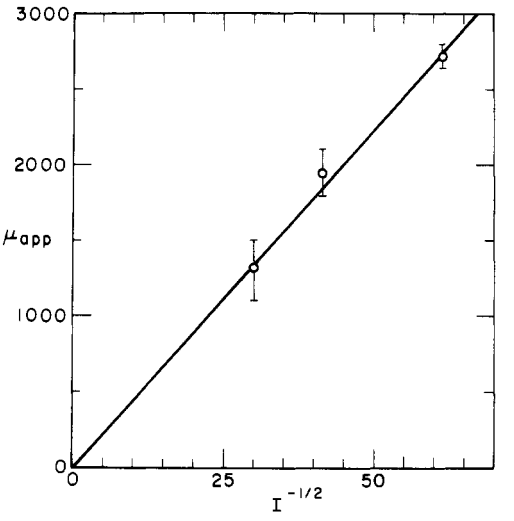

FIGURE 5: Variation of apparent dipole moment with ionic strength of the medium for the extended form. Data calculated from Figure 4.

the molecular axis, and orientation in the field is faster. Table I summarizes the observed properties. A strong decrease in $D^{(r)}$ is observed on unfolding.

Translational Diffusion Coefficients. We determined the translational diffusion constant from the observed translational correlation times. For instrumental convenience, measurements were made at 20 °C. We measured the dichroism at 20 and 7 °C to assure that no structural change occurred between those temperatures at the ionic strengths *i* (Table I) chosen for measurement. Again, a strong decrease in $D^{(t)}$ occurs upon unfolding.

Discussion

Comparison with Other Work. Table II presents an extensive, but not exhaustive, compilation of experimental determinations of the low salt unfolding behavior of nucleosomal particles. With a few exceptions, there is general agreement on a single unfolding transition for core particles, centered at about 1.1 ± 0.2 mM salt at 20 °C. Among the exceptions are the original report by Gordon et al. (1978) and the study by Harrington (1981) on samples from the same preparation. Since these two studies are in agreement, giving two transitions at ~1 and 7 mM, it is probable that the disagreement with other laboratories originates in the sample. The influence of factors such as freezing is unknown. The results of Libertini & Small (1980) give one transition, but at ~0.2 mM. Possibly the very low EDTA concentration used by those authors amplified the stabilizing influence of multivalent ions (Wu et al., 1979). Core particles of the kind used for crystallographic analysis show a single transition at ~1.2 mM (Table II).

Work on larger nucleosomes has been less extensive. The samples used by Gordon et al. (1978) and Harrington (1981)

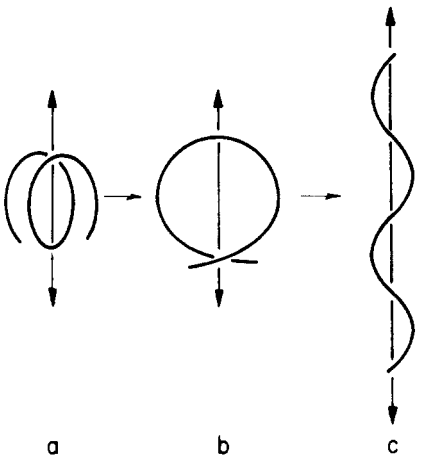

FIGURE 6: Working model for the three conformational states of 175-bp mononucleosome. (a) Compact form, 1.75–2 turns of DNA, exists under salt concentration conditions higher than 5 mM. (b) Expanded form, 1.1 turns of DNA at salt concentration between 2.5 and 1.5 mM. (c) Extended superhelical form exists at ionic strengths below 1 mM. The arrow indicates the direction of orientation in the field.

contained some material of this kind (Harrington, 1981), but it is not currently possible to assess the influence of this kind of heterogeneity of the sample. The work of Dieterich et al. (1980) reports a single transition at about 7 mM for 173- and 250-bp nucleosomes, reconstituted after fluorescent labeling. In the same study, core particles had a midpoint of 3 mM. Possibly, as suggested by Dieterich et al. (1980), counterion concentration rather than ionic strength should be used as a correlating variable. On the other hand, the difference may again originate in the sample, and it may not be just a coincidence that the 7 mM midpoint agrees with the higher salt transition reported by Gordon et al. (1978). We note that the 175-bp particle of Dieterich et al. (1980) was obtained by H1 stripping of 0.1 M KCl insoluble material, whereas our sample originates from the fraction soluble in KCl after Bio-Gel column purification.

Our results, which we have repeated by dichroism measurements a number of times, reveal two closely spaced transitions, at 1.1 and 2.9 mM ionic strength. Since the translational frictional coefficient, in contrast to the dichroism, increases in both transitions, it would probably be difficult if not impossible to resolve the unfolding into two transitions by using sedimentation measurements. Hence, our results are in accord with those of Burch & Martinson (1980), who reported a single transition midpoint of 1.6 mM for nucleosomes containing large DNA, very close to the (geometric) mean (1.8 mM) of the two transitions we observed at somewhat lower temperature (7 °C).

A Working Model. To facilitate discussion of the nature of the unfolding process, we present here a model which incorporates the results. Support for the model by the observations is discussed subsequently. Our model is similar to the one we proposed for unfolding of core particles, except that an additional unfolding step is added.

Figure 6 shows the proposed process diagrammatically. In the first step (2.9 mM), the compact particle expands into a larger disklike structure, and in the second step (1.1 mM), it elongates along the DNA superhelix axis. Hence, the names compact, expanded, and elongated are used for the three forms observed. Our model is similar to that originally proposed by Gordon et al. (1978).

Dichroism and Dipole Moment Support for the Model. The dichroism of the expanded form of 175-bp nucleosomes (−0.33) is in very good agreement with expectation based on the model proposed for the 146-bp low salt expanded form (Wu et al., 1979). In that case, the disklike particle had 0.9 turn of DNA, corresponding to 160 bp per turn, yielding a dichroism of −0.48. Keeping the same DNA periodicity of 160 bp per turn for the expanded form of 176-bp particles predicts 1.1 turns, with an expected dichroism of −0.33 (Crothers et al., 1978) as observed. Hence, the change in dichroism found between the two particles is consistent with the same DNA winding periodicity in both cases.

The finding of a salt-independent dipole moment supports a model for the expanded form which is not asymmetric enough to provide a large inducible dipole moment. The contrast with the extended form is striking in this regard.

The negative dichroism of the extended form is so large that essentially the only tenable model is one in which the DNA is stretched out along the nucleosomal superhelix axis, as we proposed for the structure resulting from ethidium binding. The superhelix pitch calculated (Crothers et al., 1978) from the dichroism is $\beta = 44°$. A highly asymmetric form is also required by the finding of a much greater induced polarization for the extended form than for the compact or expanded states.

Diffusion Coefficients. The decrease in both translational and rotational diffusion coefficients upon unfolding clearly signals an increase in molecular size during the process. Furthermore, the decrease of $D^{(r)}$ by a factor of 15, while $D^{(t)}$ decreases by less than a factor of 2, from the compact to the extended form is indicative of increased asymmetry or elongation in the extended state. Using the approach described earlier (Wu et al., 1980; Lee et al., 1981), we find the hydrodynamic data for the extended form to be consistent with a cylindrical model 470 Å long and 65 Å in diameter. With the DNA pitch angle of 44° determined from the dichroism of −0.65, we estimated 2.2 turns of DNA in the superhelix model.

Why Do Larger Nucleosomes Unfold More Extensively? The main result of this work is the observation of elongation of 175-bp nucleosomes, a transition we did not detect for core particles. The analogous process to the expansion transition in core particles is shifted from about 1.1 to 2.9 mM in 175-bp nucleosomes, followed by the elongation transition at 1.1 mM. Both of these effects indicate increased electrostatic repulsions in the particle containing more DNA, as might be expected from its increased net negative charge.

On possible explanation for observing the elongation transition only in larger nucleosomes is evident from the diagram in Figure 6. The larger nucleosomes contain 1.1 turns of DNA in the expanded state, producing extra repulsive effects in the region where there is 0.1 turn of overlap. Since overlap is absent in expanded 146-bp nucleosomes (0.9 turn), this extra driving force for elongation is absent. With our estimate of about 160 bp per turn in the expanded state, we predict that the critical DNA size for elongation should be about 160 bp. However, we also take cautionary note of the distinct difference in electrophoretic gel mobilities of the two classes of nucleosomes, greater than might have been expected given the overlap of DNA sizes. Hence, there may be some structural difference between the two nucleosome classes in addition to the difference in DNA size. Indeed, such a structural difference, and its influence on nuclease digestion, could be the origin of the difference in average DNA size and could significantly affect low salt unfolding.

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Antigenicity of Elastin: Characterization of Major Antigenic Determinants on Purified Insoluble Elastin†

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ABSTRACT: This investigation reports a systematic comparison of antibody responses to well-defined fragments of mature, insoluble elastin in order to identify antigenic regions of the molecule. Antiserum to insoluble elastin was used to screen by radioimmunoassay the serologic activity of elastin peptides purified from a thermolysin digestion of insoluble elastin. Fractions with positive antigenicity were characterized by amino acid analysis and protein sequence analysis. Our results indicate that antigenic determinants on elastin can be classified functionally into two categories: major determinants related to the cross-linking domain which show cross-reactivity between species and limited, species-specific determinants in non-cross-linked regions of the molecule.

Elastin is an important connective tissue macromolecule that imparts elasticity to elastic tissues. It is synthesized and secreted as a soluble, single-chain protein (tropoelastin) that undergoes numerous postribosomal modifications prior to organization of the elastic fiber in the extracellular space. Once secreted, tropoelastin molecules are joined covalently through chemical modification and cross-linking of specific lysyl residues to form mature, insoluble elastin.

Elastin possesses multiple, repeating structural domains which modulate its biological properties. One such domain is the cross-linking region, a polyalanine-enriched area of 30–40 amino acid residues that assumes a tight helical conformation (Gray et al., 1973; Foster et al., 1976). Since the proper helix configuration is an important determinant in correct cross-link formation, the amino acid sequence of this region is highly conserved, and cross-linking domains distributed throughout the molecule show high sequence homology. Interspersed among the cross-linking domains are regions of hydrophobic amino acids which form loose coils of β turns (Gray et al., 1973; Urry, 1974). These non-cross-linked or so-called "straight-chain" domains are thought to provide a molecular basis for the protein's elastic behavior. In contrast to the multiple cross-linking regions which exhibit compositional and structural homology, considerable sequence and conformational heterogeneity is found within the straight-chain regions.

Over the past several years, immunological techniques have become increasingly important in studies of elastin structure and biosynthesis. Antisera have been developed which are specific for elastin, but little is known concerning the antigenic determinants on the elastin molecule. This is due in part to the insoluble nature of elastin and its weak immunogenicity and because antigen–antibody complexes of elastin fragments often remain soluble (Jackson et al., 1966; Kucich et al., 1981; Mecham & Lange, 1981).

Recent studies have characterized at least two reacting antibody subpopulations in antisera to elastin: (a) antibodies which show species specificity and (b) antibodies that show

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