Chromatin Structure

EVIDENCE THAT THE 30-nm FIBER IS A HELICAL COIL WITH 12 NUCLEOSOMES/TURN*

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Sedimentation analysis has been used to compare the structure of 30-nm chromatin fibers, isolated and digested under conditions that maintain the native structure, with relaxed-refolded chromatin. The native chromatin fibers show sharp, ionic strength-dependent changes in sedimentation coefficient that are not apparent in relaxed-refolded fibers. The first transition at approximately 20 mM ionic strength reflects the organization of the 10-nm polynucleosome chain into a loose helically coiled 30-nm fiber. Between 20 and 60 mM ionic strength there is considerable interaction between nucleosomes within the coils to generate a stable helical array with 12 nucleosomes/turn. Above 60 mM ionic strength the helical coil continues to condense until it precipitates at ionic strengths slightly greater than those considered physiological, indicating that there is no end point in fiber formation. The data is incompatible with a solenoid model with 6 nucleosomes/turn and also rules out the existence of a beaded subunit structure.

Three types of models have been proposed for the structure of the 30-nm chromatin fiber, the original solenoid model of Finch and Klug (1), the superbead or nucleomer model (2, 3) and, more recently, a number of models that are all based upon a helical coil arrangement of the nucleosomes (4-10). Although many different sources of chromatin have been used it is apparent that the above models have been derived from distinctive methods of chromatin preparation. Thus, the solenoid model was proposed from work carried out on refolded chromatin, that is the native fiber was allowed to completely unfold and then re-folded in the presence of cations. Superbeads, on the other hand, are only seen in sucrose gradients or in the electron microscope when chromatin is prepared and digested at certain intermediate ionic strengths, typically 40-60 mM monovalent cation, whereas the various helical coil models have evolved from work carried out predominantly on either native chromatin or fibers that were never exposed to extremely low ionic strengths. It is conceivable, therefore, that the different models are a reflection of artifactual changes introduced into the fiber structure. Alternatively, data obtained from partially unfolded, or re-folded fibers may not be representative of the fiber in vivo. Quite clearly, to be acceptable a model derived from studies carried out in one set of conditions must be able to explain how the fiber behaves under all conditions.

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Sedimentation analysis is one of the few solution techniques that permits chromatin to be studied over a wide range of ionic strengths and has been used to provide data that is considered to support each of the above models (2-5, 8-11). In this paper we have used this technique to study the changes of both native and relaxed-refolded liver chromatin over the entire range of ionic strengths (10-170 mM monovalent cation) that effects the 10 → 30-nm fiber transition. The data is not consistent with either the solenoid or the superbead models for the structure of the 30-nm fiber, but is compatible with a helical coil arrangement containing 12 nucleosomes/turn.

MATERIALS AND METHODS

Nuclei were isolated from rat liver in 0.25 M sucrose, 50 mM Tris-HCl (pH 7.5), 150 mM KCl, 5 mM MgCl₂, and 0.2 mM phenylmethylsulfonyl fluoride as described previously (12, 13). The nuclei were resuspended at 0.8-1.0 mg of DNA/ml in 5 mM Tris-HCl (pH 8.2 at 30 °C), containing 0.2 mM phenylmethylsulfonyl fluoride together with the concentrations of monovalent cations described in the text. Micrococcal nuclease (Sigma or Pharmacia Biotechnology Inc.) at a concentration of 50 units/ml (13) was added and the nuclei digested at 30 °C for 5-15 min. The reaction was terminated by the addition of EDTA to 1 mM followed by rapid cooling in ice water. The suspension was then centrifuged at 25,000 × g for 15 min at 5 °C to generate a supernatant containing 30-40% of chromatin as soluble oligonucleosome fragments.

Sedimentation coefficients of the oligonucleosomes were determined by layering (0.5 ml of the supernatant (1-5 mg DNA units) onto a 8-35% (w/w) sucrose gradient (prepared in 5 mM Tris-HCl, pH 8.1, at 5 °C, 1 mM EDTA and the KCl concentration indicated in the legends). The gradients were centrifuged at 5 °C in a SW40 rotor at 40,000 rpm to preset ω²c values of either 2 × 10⁶ or 5 × 10⁶ radians² s⁻¹ (350 and 480 min, respectively) in a Beckman L8-70 ultracentrifuge. The gradients were fractionated into 0.7-ml fractions (15) for refractive index determination and DNA size analysis.

DNA was extracted from the gradient fractions and electrophoresed as described previously (13). Each set of gradient fractions on the gels was flanked by a series of DNA gel markers (1-kilobase and 123-base pair ladders and a HindIII digest of λ DNA, Bethesda Research Laboratories). Gels were visualized and photographed immediately after electrophoresis (13) and 8 × 10-inch negatives were produced and scanned on a Beckman DU-8 spectrophotometer. Because the relationship between log (DNA size) and distance migrated is seldom linear over large size ranges, the spectrophotometer was used solely to accurately determine the position of standard and unknown DNA bands. A standard curve was then drawn manually and the DNA size of unknown fragments was determined from this curve.

Calculation of the Data and Experimental Ratios—Sedimentation coefficients (s₂₀,₅₀) were determined for each gradient fraction, assuming a particle density of 1.5, using a computer program modified from that described by Young (14) to evaluate the expression:

\[ s_{20,50} = \int \frac{vdr}{\eta_p(c - \rho_m) - \eta_p(c - \rho_p)} \]  

where υ = viscosity, ρ = density of particle (ρ), sucrose (m), and

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RESULTS

Gradient Profiles of Chromatin Fragments—The amount of chromatin released from nuclei by micrococcal nuclease in a given time period is dependent upon the concentration of monovalent cation (13, 18) and in these experiments the digestion time was adjusted so that a minimum of 30–40% of the chromatin was solubilized. Typical optical density profiles of chromatin digests were analyzed as described in the Materials and Methods section.

The sedimentation coefficient of a chromatin oligomer is related to its mass by the Svedberg equation,

\[ \frac{S_{20,w}}{M^\alpha} = \frac{(\rho_p - \rho_w)}{18 \eta_s}, \]

which defines the sedimentation coefficient in terms of the physical characteristics of the macromolecule. Thus the rate of sedimentation of a particle is proportional to the square of its diameter, \( d^2 \), and for a spherical particle with volume \( V = \frac{4}{3}\pi r^3 \), sedimentation is therefore proportional to \( V^{1/2} \). For nonspherical macromolecular polymers this formula is generalized to \( a \) with \( \alpha \) being a function of the shape of the macromolecular complex (16, 17). For example, a compact cluster of oligomers approaches the theoretical maximum value of \( \alpha = 0.667 \), whereas a more open, flexible coil or chain of oligomers, such as polynucleosomes, has a value of 0.5-0.55. A random coil on the other hand has a value of \( \alpha = 0.2-0.3 \) (16). Since \( \alpha \) can be evaluated from double logarithmic plots of \( S_{20,w} \) versus \( M \) this approach can be used to gain insight into the changes in structural organization that occur as the 10-nm chromatin fiber is resolved into its 30-nm subunits. The sedimentation behavior observed above is independent of the ionic strength at which the samples are run.

FIG. 1. Gradient profiles of chromatin oligomers sedimented to \( \omega^2 t \) values of \( 5 \times 10^{11} \) (A) or \( 2 \times 10^{11} \) (B). Gradients contained KCl to give an ionic strength of either 10 (---) or 100 mM (---). The number \( \omega^2 t \) refers to the position of mononucleosomes and sedimentation is from left to right.

Size analysis of the DNA in fractions from long and short runs are shown in Fig. 2. The data in Fig. 2A confirmed that each successive peak in the \( A_{260} \) profile corresponded to an increment of 1 in oligomer size. For the short runs (Fig. 2B) each fraction contained a discrete subset of oligomer sizes for which a mass average could be determined by densitometry. In addition, this gel also shows that the fast-sedimenting material consisted of long nucleosome oligomers rather than aggregates of smaller particles.

Sedimentation Analysis of Long Oligomers—The ionic strength-dependent changes in chromatin fiber structure were studied on both native chromatin in the process of unfolding (Fig. 3A) and chromatin that had been previously relaxed by exposure to low ionic strength buffers (Fig. 3B). In these experiments the ionic strength in the gradient tube was identical to that of the digestion buffer. The data is presented as double logarithmic plots of \( S_{20,w} \) versus \( M^\alpha \). Exposure to lower ionic strengths displaced the line downwards which indicated that the 30-nm fiber was becoming less compact. In addition, there were pronounced changes in the slope of these lines (i.e. the value of \( \alpha \) in the above equation) particularly at higher ionic strengths. At all ionic strengths greater than 20 mM the lines converged and intersected at a point corresponding to 11–12 nucleosomes.

The sedimentation behavior observed above is independent of the ionic strength at which the samples are run. The data in Fig. 3A and 3B shows that the sedimentation coefficient of the chromatin oligomers is dependent upon the concentration of monovalent cation (13, 18) and in these experiments the digestion time was adjusted so that a minimum of 30–40% of the chromatin was solubilized. Typical optical density profiles of chromatin digests were analyzed as described in the Materials and Methods section.

The sedimentation coefficient of a chromatin oligomer is related to its mass by the Svedberg equation,

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which defines the sedimentation coefficient in terms of the physical characteristics of the macromolecule. Thus the rate of sedimentation of a particle is proportional to the square of its diameter, \( d^2 \), and for a spherical particle with volume \( V = \frac{4}{3}\pi r^3 \), sedimentation is therefore proportional to \( V^{1/2} \). For nonspherical macromolecular polymers this formula is generalized to \( a \) with \( \alpha \) being a function of the shape of the macromolecular complex (16, 17). For example, a compact cluster of oligomers approaches the theoretical maximum value of \( \alpha = 0.667 \), whereas a more open, flexible coil or chain of oligomers, such as polynucleosomes, has a value of 0.5-0.55. A random coil on the other hand has a value of \( \alpha = 0.2-0.3 \) (16). Since \( \alpha \) can be evaluated from double logarithmic plots of \( S_{20,w} \) versus \( M \) this approach can be used to gain insight into the changes in structural organization that occur as the 10-nm chromatin fiber is resolved into its 30-nm subunits. The sedimentation behavior observed above is independent of the ionic strength at which the samples are run.

FIG. 2. Agarose gels of DNA fragments isolated from gradient fractions. A, fractions 4–18 from a long \( \omega^2 t = 5 \times 10^{11} \) radians/s run. B, fractions 2–17 from a short run \( \omega^2 t = 2 \times 10^{11} \) radians/s. Both gradients contained monovalent salt (K+) to 60 mM ionic strength. The 123-base pair (bp) ladder contains integer multiples of the lowest band (123 base pairs), the 1-kilobase (kb) ladder has predominant peaks at 506, 1,018, 1,635, 2,036, 3,054, 4,072, and 5,080 base pairs and the \( \lambda \) digest has bands at 560, 2,000, 2,900, 4,400, 6,700, 9,400, and 23,100 base pairs.
of the extent of digestion at any given ionic strength. Thus although the size distribution of the fragments decreases (see Fig. 2 of Ref. 20) as digestion proceeds, the sedimentation properties of oligomers of a given size remain the same (data not shown). Since as much as 80–85% of chromatin can be released during these prolonged digestions the data reflects the sedimentation behavior of bulk chromatin rather than that of specific subsets released differentially at the various ionic strengths.

When similar experiments were performed on chromatin that was relaxed by exposure to an ionic strength of 5 mM prior to digestion and sedimentation analysis the results were quite different (Fig. 3B). The lines were displaced upwards as the fibers became more compact with increasing ionic strength, but the slope of the lines showed only modest increases and no point of convergence was evident.

The differences between refolded and native chromatin were more evident in a plot of $s_{20,w}$ versus ionic strength (Fig. 4). Long oligomers ($n = 20–60$) of native chromatin produced triphasic curves with pronounced breaks at 60 and 20 mM ionic strengths (Fig. 4A). For oligomers consisting of 6 or 10 nucleosomes the transition at 60 mM was absent and their sedimentation coefficients were independent of ionic strength above 20 mM. Oligomers of refolded chromatin, on the other hand, behaved quite differently (Fig. 4B) with particles containing from 10 to 60 nucleosomes showing a linear increase in $s_{20,w}$ with ionic strength. This latter data is similar to that described by Butler and Thomas (11) for refolded liver chromatin.

**Significance of the Changes in the Value of the Exponent, $a$—**Values of $a$ were obtained by regression analysis of data obtained as described in the legend to Fig. 3 for both native and refolded chromatin oligomers. The changes in $a$ as a function of ionic strength are shown in Fig. 5. Native chromatin, isolated and digested at physiological ionic strengths (~170 mM) produced chromatin fragments with a value of $a$ of 0.63 typical of highly compacted oligomers. As the chromatin was exposed to progressively lower ionic strengths there was a linear decrease in $a$ towards values typical of a more open helical coil (Fig. 5A). Further decreases in ionic strength from 60 to 20 mM produced only a small change in the value of $a$, but below 20 mM the values decreased markedly towards values typical of a random coil. Also included in Fig. 5A are values of the exponent $a$ for a chromatin sample (52% of total nuclear chromatin) which was isolated and digested at 120 mM ionic strength and then adjusted to, and centrifuged at, lower ionic strengths of 80, 60, 25, and 10 mM. The close correspondence between these values and those obtained as described above where the ionic strength was lowered prior to digestion eliminates the possibility that digestion at different ionic strengths releases subsets of chromatin with different sedimentation properties. The equivalent data for refolded chromatin is shown in Fig. 5B. There was a sharp increase in the value of $a$ as the ionic strength was increased to 20 mM indicating that the relaxed oligomers were taking on a more organized higher order structure. However, above 20 mM there was only a gradual increase in slope with no indication of a transition at 60 mM.

Superimposed on the native chromatin data in Fig. 5A is the pattern of change in sensitivity of the fiber to a 5-min incubation with micrococcal nuclease. This data is similar to that observed previously by us (13) with additional data at lower ionic strengths. These changes in sensitivity complement the structural transitions that were indicated by the...
changes in the value of $a$. Thus as the fiber folded from the relaxed 10-nm fiber into a helical coil there was a decrease in nuclease sensitivity. Between 20 and 60 mM there was an increase in nuclease sensitivity which appears to be related to structural rearrangements of the linker DNA (13) as the nucleosomes become organized into a helical array. Finally, as the fiber underwent further compaction above 60 mM ionic strength there was a concomitant decrease in the accessibility of the fiber to the nuclease.

**Sedimentation Analysis of Short Oligomers**—Sedimentation coefficients of monomers--dodecamers of native chromatin exposed to various ionic strengths were estimated directly from their peak positions in short runs (Fig. 6A). The sedimentation coefficients of the oligomers were relatively insensitive to decreases in ionic strength down to 20 mM. Below this concentration there were sharp decreases in the $s_{20w}$ value indicating a substantial change in the compaction of the oligomers. Furthermore, the relationship between $s_{20w}$ and $n$ was linear in the range 1–12 nucleosomes at all ionic strengths with no indication of a break in the curve at 6 nucleosomes. Indeed, when the data for short oligomers is combined with that for longer fragments (Fig. 6B) then a pronounced change in slope becomes evident at 12 nucleosomes particularly at lower ionic strengths.

**Discussion**

Rate zonal centrifugation has a number of advantages over the more conventionally used analytical ultracentrifuge for the analysis of the sedimentation characteristics of chromatin. It permits the simultaneous analysis of the behavior of a whole range of oligomer sizes at as many as 6 different ionic strengths. The data generated is, therefore, a function of the behavior of the whole population of molecules in solution and not just an average of all the molecules in the digest, a problem that is often encountered in solution studies (21). Furthermore, for small oligomers ($n \leq 12$) the $s_{20w}$ values are determined for each oligomer directly rather than using the mass average of a previously fractionated sample (11). In our hands, mass averages could never be accurately determined for mixtures of small oligomers. In addition, sedimentation analysis can be carried out over the entire range of ionic strengths that effect the $10 \leftrightarrow 30$-nm transition, especially at the higher, more physiological ionic strengths (~150 mM).

Quite clearly, refolded oligomers do not have the same sedimentation characteristics as native fibers. Thus while exposure to increasing ionic strength promotes compaction of the relaxed polynucleosome chain, the 20 and 60 mM transitions are either less marked or absent and the refolded fibers do not appear to reach the same level of fiber condensation as native fibers (compare the values of $a$ in Fig. 5). Therefore, while we were able to reproduce the sedimentation data of Butler and Thomas (11) for refolded chromatin we believe that it cannot be used in support of a solenoid model for the native 30-nm fiber.

The folding and structural organization of the 30-nm fiber can be conveniently examined in 3 stages based upon the changes in the value of $a$ (Fig. 5A). The value of $a$ decreases rapidly below 20 mM towards values consistent with a random coil of polynucleosomes (16). Electron micrographs of chromatin at these ionic strengths also showed a relaxed fiber (5, 22, 23). At approximately 20 mM ionic strength the nucleosomes become organized into a loose, irregular helical array (5, 22) with fiber dimensions that already approximate those of the native 30-nm fiber. A value for $a$ of 0.45 indicates that this structure also exists in solution. The value of $a$ increases only slightly as the ionic strength changes between 20 and 60 mM. However, there are indications that there is considerable internal organization of the fiber at this time, presumably induced by charge neutralization of linker DNA. First, the fiber becomes less sensitive to exogenous nuclease at sites within each loop (13, 20). This leads to the preferential release of oligomers containing approximately 12 nucleosomes (Figs. 1 and 2, see also Ref. 20). This observation forms the basis of the nucleomer or superbead models (2, 3, 19) but is only observed at these ionic strengths. Electron micrographs of the fiber also show considerable irregularity at this ionic strength (5, 22). Second, there is a concomitant increase in the sensitivity to proteases of the extended "arms" of histone H1 (24–26). Third, there are changes in the birefringence properties of the fiber indicating a change in the orientation of the nucleosomes relative to the fiber axis (24). All of this evidence suggests that in this ionic strength range the nucleosomes become organized into loops with about 12 nucleosomes in a loop. Each loop is stabilized by H1-H1 interactions and the linker DNAs between nucleosomes within the loop are protected from exogenous nuclease which accounts for the release of fragments containing predominantly 12 nucleosomes.

Above 69 mM ionic strength the fiber looks relatively smooth in the electron microscope (5, 22) but since the value of $a$ continues to increase it appears that in solution the helical coil continues to compact until the fragments eventually precipitate. Thus, while electron micrographs of liver chromatin (22) indicate no overall change in fiber dimensions above 60 mM ionic strength, it cannot be concluded (11, 22)
that the fiber observed in vitro at 60 mM represents the native fiber. This continued increase in the value of \( a \) supports the view (5) that the mass per unit length continues to increase with increasing ionic strength. Thus while there is general agreement that the mass per unit length at 60 mM ionic strength is equivalent to 6 nucleosomes/11 nm (5, 22), this does not reflect the maximum compaction of the 30-nm fiber. At physiological ionic strengths, the mass per unit length is equivalent to approximately 12 nucleosomes/11 nm (5), a value incompatible with a contact helix containing only 6 nucleosomes/tturn. All of these lines intersect at an oligomer size of 12, indicating that this is the minimum number of nucleosomes required to generate one stable turn of the helix. Oligomers containing less than 12 nucleosomes cannot undergo this compaction and are unaffected by increases in ionic strength above 20 mM. In addition, in a plot of \( \log (s_{20,w}) \) versus \( \log (a) \) for the entire size range of oligomers (Fig. 6B) there is a pronounced break in the line at \( a = 12 \). This decrease in the value of \( a \) is consistent with oligomers containing more than the number of nucleosomes in a turn, folding to form and extend a helical coil or rod. This is particularly evident at low ionic strengths when the coil is very extended. Although this data could also be compatible with a solenoid with 6 nucleosomes/tturn if one assumed that 2 turns of the helix were necessary to form a stable unit, the continued increase in the value of the exponent \( a \) and the increased mass per unit length values described above render this a less likely possibility. A helical coil with 12 nucleosomes/tturn is the simplest model that is compatible with most of the biophysical data obtained from studies carried out at various ionic strengths. Once the fiber is formed, its diameter would be expected to be relatively independent of ionic strength as observed by Williams et al. (10), whereas the pitch would decrease with increasing ionic strength producing the observed increases in the mass per unit length (5) and value of the exponent \( a \). The fiber continues to compact in vitro until precipitation occurs supporting the contention that there is no end point to fiber formation (27). Low angle x-ray scattering has been used (10, 28) to try to deduce the pitch of the helix from the meridional banding pattern. Although Widom and Klug (28) interpreted their data in terms of a pitch of 11 nm, more recent studies (10) indicate that the value is somewhat higher (24-27 nm), which is very close to the values derived for a simple helical coil by Fulmer and Bloomfield (9). Quite clearly, it is mandatory to obtain accurate values for the pitch of the 30-nm fiber as a function of ionic strength. Although sedimentation analysis can yield information concerning the folding of the 10-nm polynucleosome chain into the 30-nm fiber and give some indication of overall fiber shape it cannot distinguish between a simple one-start helical coil and the more complex helical ribbon (5) or cross-linker models (10). However, the data presented in this and the accompanying paper (20) show clearly that a dodecamer is a stable intermediate in fiber folding and it is not immediately obvious how the more complex models accommodate this observation. In summary, there is now a growing body of evidence from both solution techniques (9, 20, 29, 30, this study) and electron microscopy (5) of chromatin isolated at physiological ionic strengths that the 30-nm fiber is generated by the helical coiling of the 10-nm polynucleosome chain into a helix with about 12 nucleosomes/tturn. The changes in sedimentation behavior as the fiber unfolds are consistent with all the changes in shape of the fiber observed in the electron microscope. In addition, the data confirms that so-called “superbead” profiles are a reflection of intermediate stages in fiber folding (13, 20). Furthermore, it appears that higher levels of compaction than are allowed by a contact helix (solenoid) with 6 nucleosomes/tturn are achievable at physiological ionic strengths. Finally, although we have referred to the higher order chromatin fiber as the 30-nm fiber it must be recognized that in vitro the diameter of the fiber may well exceed 40 nm (9, 21).

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