Properties of Nuclease-resistant Fragments of Calf Thymus Chromatin

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SUMMARY

Nuclease-resistant chromatin fragments have been isolated on the basis of their insolubility in 0.15 M NaCl after limited digestion of calf thymus chromatin with micrococcal nuclease. Such fragments exhibit an unusual circular dichroism spectrum in the range from 200 to 300 nm, which is unlike that of B form DNA or undigested chromatin and most closely resembles that of C form DNA. Electric dichroism and sedimentation velocity measurements suggest that the DNA in these fragments is folded into a compact, perhaps superhelical, configuration. The average A_{290} nm : A_{230} nm ratio for six preparations was 1.27 ± 0.02, indicating a well defined composition of approximately 1.85 mg of protein per mg of DNA.

Chromatin isolated from nuclei of higher organisms consists primarily of basic histone proteins and DNA, with an additional, relatively small amount of more acidic proteins. Despite the approximate equivalence of numbers of histone-basic amino acids and nucleotide phosphate groups, studies of the binding of polylysine to chromatin indicate that histones are not uniformly distributed along the DNA chain (1, 2). Recently, various endonucleases have been used to cleave, and subsequently fractionate, chromatin into histone-rich and histone-poor fragments (1, 3-5). Summarizing briefly, Marushige and Bonner (3) have shown that limited digestion of chromatin with DNase II produces two fractions which are separable on the basis of their solubilities in 0.15 M sodium citrate. The minor, salt-soluble fraction is relatively poor in histones, rich in non-histone proteins, and has been implicated as the template-active portion of chromatin. The major, salt-insoluble fraction is correspondingly rich in histone, poor in non-histone proteins, and template-inactive. Under more optimal conditions, other nucleases, such as DNase I and staphylococcal nuclease, readily digest 50 to 75% of chromatin DNA (1, 4, 5). Mirsky (5) has shown that the final extent of digestion depends strongly upon enzyme concentration, incubation time, and, to some extent, upon the method of chromatin preparation. On the basis of such results, Itzhaki (4) and Mirsky (5) have proposed that virtually all chromatin DNA is associated with protein, but the extent or tightness, or both, of the association, and hence the accessibility to nuclease(s), is not uniform.

Up to this time, little effort has been directed toward determining the properties of what may be classed as the "nuclease-resistant" portions of chromatin. Below, we report on some preliminary physical studies of such chromatin fragments.

METHODS AND MATERIALS

Chromatin was prepared from calf thymus according to the procedure of Maurer and Chalkley (Ref. 6; Method V), in which nuclei are initially isolated in 0.25 M sucrose-3 mM CaCl₂-5 mM Tris, pH 7.35. This crude chromatin was further purified by sucrose density centrifugation (7). In some cases chromatin was sheared with a VirTis homogenizer for 90 s at 40 volts. Such sheared chromatin typically had ~ν = 28 S to 32 S in 10 mM Tris, pH 8, an A_{230} nm : A_{230} nm ratio of 1.40 to 1.45, and contained 1.0 to 1.1 mg of histone per mg of DNA.

DNA concentrations were determined with diphenylamine (8) or from A_{260} nm, assuming E_{1%} = 6.8 × 10² M⁻¹. Histone concentrations were determined by the Lowry procedure as described by Tuan and Bonner (9). Protein concentrations of the chromatin fragments were sometimes estimated from the absorbance at 280 and 230 nm (9), assuming that extinction coefficients for histone and non-histone proteins are the same at 290 nm.

Digestions of chromatin with micrococcal nuclease (Worthington) were carried out in 5 mM sodium phosphate-2.5 × 10⁻⁸ M CaCl₂, pH 6.7, at 37° (1). Chromatin concentrations were approximately equivalent to 0.25 mg of DNA per ml; the nuclease concentration was 5 µg per ml. Digestion was terminated after a given amount of time by addition of enough cold 0.2 M NaCl-6 mM EDTA, pH 7.3, to give a final sodium ion concentration of 0.15 M. At this sodium ion concentration, native chromatin is minimally soluble (10), hence, it was expected that the undigested portions of chromatin would precipitate at this point. The precipitate that formed was removed by centrifugation at 25,000 rpm for 30 min in a Spinco No. 30 rotor. Supernatant fractions obtained from this centrifugation are referred to as "high salt-soluble" or S fractions. The pellets were washed three times to remove nuclease by resuspending in 0.126 M NaCl-8 mM EDTA, pH 7.3, and centrifuging as before. The washed pellets were then redissolved by stirring overnight in

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0.01 M Tris-cacodylate, pH 8.0, at 4°C. Any remaining insoluble material was removed by centrifugation. Soluble fractions thus obtained are referred to as "low salt-soluble" or PS fractions. Pellets from this centrifugation are referred to as PEL fractions.

Ultraviolet absorption spectra were recorded on a Cary 15; circular dichroism spectra were measured on a Jasco-Durrum J-10. Electric dichroism measurements were made with an instrument constructed in this laboratory as described previously (11). All electric dichroism data were taken at 15°C in 1 x 10^{-4} M Tris-cacodylate, pH 8.0. Sedimentation coefficients were determined with a Spinco model E ultracentrifuge equipped with scanner optics set at 265 nm and were corrected to n_0c by standard methods.

RESULTS AND DISCUSSION

The yields and pertinent spectral data for S, PS, and PEL fractions from sheared chromatin after digestion for 10, 20, 30, and 240 min are summarized in Table I. Within 10 min, much of the sheared chromatin is rendered soluble in 0.15 M NaCl. The nature of this high salt-soluble material (S fraction) evidently changes with increasing digestion times, as is illustrated by the progressive increase in \( A_{260} \text{nm} : A_{300} \text{nm} \) ratios and extinction coefficients at 258 nm. Circular dichroism spectra of S fractions from 10- to 30-min digests retained an intense positive band resembling that of B-form DNA between 260 and 300 nm (see Fig. 1). This result suggests that these fractions still contained fairly long DNA segments. In the case of S1 this was confirmed by sedimentation velocity measurements which showed a fast moving boundary, corresponding to 24% of the sample, with a medium \( s_{20, w} = 15 S \). The remaining absorbing material remained at the meniscus.

Prolonging the digestion past 10 min resulted in an increase in low salt-insoluble (PEL) material at the expense of both the S and PS fractions. Itzhaki (4) also noted a decrease in supernatant DNA concentration and an accumulation of insoluble material after prolonged digestion and has suggested that this material results from aggregation of released protein and other chromatin fragments. The low \( A_{260} \text{nm} : A_{300} \text{nm} \) ratios of the PEL fractions dissolved in 1% sodium dodecyl sulfate confirm that these fractions have a high protein content relative to chromatin.

Qualitatively similar results were obtained upon digestion of chromatin which had not been previously sheared. The digestion was not quite as rapid in this case. A larger portion of the S fractions sedimented rapidly; approximately 25% of the original DNA was recovered in the PS fraction after digestion for 20 min, and little DNA was lost in the PEL fractions.

The primary aim of this preliminary study was to determine some properties of the nuclease-resistant portions of chromatin found in the PS fractions. Table II summarizes most of the data measured on several preparations of these fractions. In contrast to the S and PEL fractions, the \( A_{260} \text{nm} : A_{300} \text{nm} \) ratios for the PS fractions are essentially independent of digestion time. This result suggested that the PS material has a fairly well defined composition, estimated as 1.85 ± 0.04 mg of protein per mg of DNA from the average of the \( A_{260} \text{nm} \) and \( A_{300} \text{nm} \) values of the six preparations (9). This ratio is significantly higher than the protein to DNA ratio observed for whole chromatin.

The circular dichroism spectra of the PS fractions are very unusual, as is illustrated in Fig. 1 by the comparison of their spectra with those of free DNA, sheared chromatin, and a typical S fraction. When the PS fractions were treated with 2 M NaCl or 6 M urea to dissociate proteins, the resulting circular dichroism spectra were very similar to those of DNA in the same solvents as is shown in Fig. 2. Such behavior indicates that proteins are responsible for maintaining DNA in a unique configuration.

### Table I

Yields and spectral data for fractions obtained upon digestion of sheared chromatin

| Digestion time | Fraction | Percentage of initial DNA | \( A_{260} \text{nm} : A_{300} \text{nm} \) | \( E_{258} \text{nm} \) |
|---------------|---------|---------------------------|---------------------------------|-----------------|
| 10 min        | S       | 71.5                      | 1.75                            | 8.69            |
|               | PS      | 19.5                      | 1.23                            | 7.46            |
|               | PEL     | 4.4                       | 0.73                            |                 |
| Total         |         |                           |                                 | 98.4            |
| 20 min        | S       | 60.8                      | 2.05                            | 9.65            |
|               | PS      | 14.1                      | 1.32                            | 7.94            |
|               | PEL     | 19.6                      |                                 |                 |
| Total         |         |                           |                                 | 94.5            |
| 30 min        | S       | 61.3                      | 2.15                            | 10.2            |
|               | PS      | 5.2                       | 1.27                            |                 |
|               | PEL     | 28.5                      | 1.05                            |                 |
| Total         |         |                           |                                 | 98.0            |
| 240 min       | S       | 71.8                      | 2.39                            | 11.1            |
|               | PS      | none                      |                                 |                 |
|               | PEL     | 26.5                      | 0.61                            |                 |
| Total         |         |                           |                                 | 98.3            |

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* PEL fractions were dissolved in 0.01 M Tris-1% sodium dodecyl sulfate, pH 8.

### Table II

Properties of PS fractions from sheared and unsheared chromatin

| Sample | Digestion time | \( A_{260} \text{nm} : A_{300} \text{nm} \) | \( \langle \theta \rangle_{260} \text{nm} \) | \( \langle \theta \rangle_{300} \text{nm} \) | \( s_{20, w} \text{S} \) | \( \Phi_{	ext{PS}} \) (DNA) |
|--------|----------------|---------------------------------|-----------------|-----------------|-----------------|------------------|
| Sheared chromatin | | | | | | |
| PS1    | 10             | 1.23                            | -776            | 776             | 1521            | 18.6             |
| PS2    | 20             | 1.32                            | -709            | 794             |                 |                  |
| PS3    | 30             | 1.27                            |                 | 6.3             | 18.0            | 6.0              |
| Unsheared chromatin | | | | | | |
| PS1    | 10             | 1.27                            | -386            | 1521            | 18.6            | 6.3              |
| PS2    | 13             | 1.25                            | -419            | 1548            | 18.0            | 6.0              |
| PS3    | 20             | 1.36                            | -565            | 1182            | 16.5            | 5.8              |

* Molecular ellipticities are reported as deg cm² per decimole of phosphorus.

* Determined in 0.01 M Tris-cacodylate, pH 8.

* Determined after digestion with pronase (see text) in 0.2 M NaCl and 0.01 M Tris-cacodylate, pH 8.
FIG. 1. Circular dichroism spectra of A, commercial calf thymus DNA in 0.126 M NaCl-0.008 M EDTA, pH 8; B, sheared calf thymus chromatin in 0.01 M Tris-cacodylate, pH 8; C, high salt-soluble fraction (SI) from sheared chromatin digested for 10 min, measured in 0.126 M NaCl-0.008 M EDTA, pH 8; D, low salt-soluble fraction (PS2) from unsheared chromatin digested for 13 min, measured in 0.01 M Tris-cacodylate, pH 8; E, low salt-soluble fraction (PS1) from sheared chromatin digested for 10 min, same conditions as D; ---, presumed C form DNA film taken from data of Tunis-Schneider and Maestre (12).

in these chromatin fragments. The spectra of PS fractions from sheared chromatin are strikingly similar to that reported by Tunis-Schneider and Maestre (12) for lithium-DNA films at 72 to 95% relative humidity (also shown in Fig. 1). This circular dichroism spectrum has been attributed to C form DNA on the basis of x-ray studies of DNA under the same conditions. Similar spectra have been reported for DNA in ethylene glycol solutions by Nelson and Johnson (13), who also point out that DNA can be more tightly packed in the C form than in the A or B forms. It is also of interest to mention that Hanlon and Johnson (14) have calculated that the circular dichroism spectrum of sheared chromatin is consistent with 60 to 70% of the DNA being in the C form and 30 to 40% in the B form. Our low yields (about 20 to 25%) of the PS fractions are not necessarily inconsistent with this prediction, since some of the potential PS material may be digested or may be too small to sediment under the conditions used for isolation. The differences between the circular dichroism spectra of PS fractions from sheared and unsheared chromatin probably result from contamination of the latter material with some B form DNA.

The linear dichroism of the PS fragments oriented in electric fields is considerably lower at all field strengths than that of either low molecular weight DNA or sheared chromatin (see Fig. 3). Calculations based on curve-fitting procedures described previously (15) show that the limiting dichroism of the PS fractions cannot be significantly greater than -0.2, much lower than the limiting dichroism observed for low molecular weight DNA of -1.35 (15). This low limiting dichroism must result either from tilting of the DNA bases away from the perpendicular to the double helix axis or from folding of the chain in a manner which prevents orientation with the field. The first possibility seems unlikely because of the large tilt (about 33°) required to account for the low dichroism. Folding of the DNA chain could be irregular or regular, as in a superhelix. A limiting dichroism of -0.2 is consistent with a superhelix whose pitch to radius ratio is approximately 5.4 (16). This ratio is somewhat higher than those proposed on the basis of x-ray studies of whole chromatin (17, 18).

The suggestion that the DNA in these chromatin fragments is compactly folded is supported by the high sedimentation co-
efficients and low rotational relaxation times (measured from the decay of the electric dichroism) that we observe. While the heterogeneity of the PS fractions precludes any detailed hydrodynamic analysis, it is possible to show that the results are inconsistent with a rigid, rodlike particle in which protein is bound to an extended DNA molecule. For example, the median $s_{20, w}$ of the DNA from the PS2 fraction from unsheared chromatin is about 6.0 S (see Table II); this corresponds (19) to a median molecular weight of 200,000 and an extended length, in C form, of about 1000 Å. Since there are about 1.9 mg of protein per mg of DNA in the particles, an average particle weight of 560,000 can be calculated. A minimal particle volume (neglecting hydration) can then be estimated if the partial specific volume of the nucleoprotein is known. This was estimated as 0.69 cm$^3$ per g from values given for DNA and calf thymus chromatin (20) and histones (21). The volume so calculated yields a diameter of 30 Å for a 1000 Å-long cylinder. This is surely too small, since hydration has been neglected. Using these dimensions, together with the equations of Broersma (22) for translational and rotational frictional coefficients of cylinders, we predict an $s_{20, w}$ value of 17.5 S and a rotational relaxation time of 18.5 μs. The observed $s_{20, w}$ is much larger, and the discrepancy with the predicted value would be even greater if hydration were taken into account. While we cannot obtain accurate values for the dichroism relaxation time, since it approaches the resolution time of our instrument, the observed value cannot be greater than 7 to 8 μs. Again, taking hydration into account would increase the discrepancy. Thus, the hydrodynamic data indicate that the DNA must be folded or supercoiled to yield a compact structure, in agreement with the conclusion drawn from the electric dichroism values.

REFERENCES

1. Clark, R. J., and Felsenfeld, G. (1971) Nature New Biol. 229, 101
2. Itzhaki, R. F. (1970) Biochem. Biophys. Res. Comm. 41, 25
3. Marushige, K., and Bonner, J. (1971) Proc. Nat. Acad. Sci. U. S. A. 68, 2941
4. Itzhaki, R. F. (1971) Biochem. J. 125, 221
5. Marushige, K. E. (1971) Proc. Nat. Acad. Sci. U. S. A. 68, 2945
6. Mauger, H. R., and Chalkley, R. G. (1967) J. Mol. Biol. 27, 431
7. Bonner, J., Chalkley, R. G., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch H., Olivera, B., and Widholm, J. (1968) Methods Enzymol. 12B, 3-64
8. Burton, K. (1968) Methods Enzymol. 12B, 163-165
9. Tuan, D. Y. H., and Bonner, J. (1969) J. Mol. Biol. 45, 50
10. Frederick, E. (1971) in Histones and Nucleohistones (Phillips D. M. P., ed) p. 145, Plenum Press, New York
11. Allen F. S., and Van Holde, K. E. (1970) Rev. Sci. Instrum. 41, 311
12. Tunis-Schneider, M. J. B., and Maestre, M. F. (1970) J. Mol. Biol. 55, 521
13. Nelson, R. G., and Johnson, W. C. (1970) Biochem. Biophys. Res. Commun. 41, 311
14. Itano, S., and Johnson, R. S. (1971) Fed. Proc. 30, 1096
15. Ding, D. W., Rill, R. L., and Van Holde, K. E. (1972) Biopolymers 11, 2109
16. Rill, R. L. (1972) Biopolymers 11, 1929
17. Pardon, J. F., Wilkins, M. H. F., and Richards, B. M. (1967) Nature 215, 505
18. Bram, S., and Ris, H. (1971) J. Mol. Biol. 55, 325
19. Eigner, J., and Dott, P. (1965) J. Mol. Biol. 12, 549
20. Zubiay, G., and Dott, P. (1959) J. Mol. Biol. 1, 1
21. Edwards, P. A., and Shooter, K. V. (1969) Biochem. J. 114, 227
22. Broersma, S. (1960) J. Chem. Phys. 32, 1626, 1632
