Conformational state of DNA in chromatin subunits. Circular dichroism, melting, and ethidium bromide binding analysis.

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ABSTRACT

This study compares some physical properties of DNA in native chromatin and mono-, di-, trimucleosomes obtained after mild micrococcal nuclease digestion. Melting curves and derivatives are shown to be very similar from one sample to another although a shift from 79 to 81°C is observed between the mainly monophasic peak of multimers and chromatin. Careful analysis of the positive band of the circular dichroism spectra shows the appearance of a shoulder at 275nm, the intensity of which increases from the mono- to the di- and trimucleosome. This shoulder is maximum for native chromatin. At the same time binding isotherms of ethidium bromide are characterized by two highly fluorescent binding sites for all the samples but the product KN of the apparent binding constant of the higher affinity binding sites by the apparent number of these sites increases from the mono- to the di- and trimucleosome. There again the value is maximum for native chromatin. Such results strongly suggest that the native state of chromatin requires something more than the indefinite repeat of an elementary subunit.

INTRODUCTION

The subunit structure of chromatin is presently well established, and much evidence suggests that it is a general feature of chromatin issued from a large variety of eukaryotic cells.

Although SAHASRABUDDE and VAN HOLDE (1) referred to the "P S particles" as the nuclease resistant fragments in chromatin, they assumed such fragments to be discrete, protein rich region in chromatin. The idea that such particles could be a repeating unit constituting the basic structure of the chromatin fiber became strongly suggested by the work of HEWISH and BURGOYNE (2).

Studying on the digestion product of chromatin by an endogeneous mammalian nuclease, these authors were able to show that the DNA extracted from this digested product displayed discrete bands under polyacrylamide gel electrophoresis, the molecular weights of which are integral multiples of a smallest single unit of 200 base pairs. NOLL (3) further confirmed and im-
proved this result by using micrococcal nuclease, and he described the isolation procedure of the discrete particles either as mono- or multinucleosomes, using an isokinetic sucrose gradient.

Concurrently, definitive evidence for the reality of such a repeating substructure was provided by electron microscopy on integral chromatin (4) (5) (6) or on isolated nucleosomes (7) (8).

From another point of view, using crosslinking agents and looking at the resulting effect on histones (9) (10) (11) (12) (13) (14), it became evident that the four histones, H\textsubscript{2A}, H\textsubscript{2B}, H\textsubscript{3}, H\textsubscript{4}, are very close to one another in the native state of chromatin. THOMAS and KORNBERG (15) proposed that the histones form a protein core or an octamer, with two of each of these histone fractions. Histone H\textsubscript{1} does not seem to be implicated in this core, but is associated with the nucleosome. On the other hand, DNA in the nucleosome is highly accessible to both nuclease digestion (DNase I) (16), and ethidium bromide intercalation (17). All these results lead to the actual representation of the nucleosome consisting of a protein core with the DNA wrapped around it. This model was also supported by the neutron diffraction studies of BALDWIN et al. (18). However, the arrangement of nucleosomes in the chromatin is still not clear and is of much controversy, (7) and (19) or (4) and (6).

In the present study, we have investigated the physical properties of DNA in mono-, di-, tri-nucleosomes obtained by sedimenting the digested chromatin on an isokinetic sucrose gradient and compared them with those from native chromatin. While the melting behaviors of the individual fractions are very similar, circular dichroism and ethidium bromide binding properties show differences from one sample to another and from native chromatin. These data suggest that the native state of chromatin requires something more than the subunit entity indefinitely repeated.

**MATERIAL AND METHODS**

Native chromatin was obtained from rat liver nuclei extracted according to HEWISH and BURGOYNE (2) and suspended at a concentration of 1.5 to 2x10\textsuperscript{8} nuclei/ml in buffer A plus 0.34 M sucrose. Chromatin extraction was essentially performed as described by NOLL et. al. (20), 15 units of micrococcal nuclease (WORTHINGTON) was added per ml of this suspension activated by 1mM CaCl\textsubscript{2} at 37\textdegree C. The digestion was stopped 20 seconds later by adding 1mM EDTA and chilled quickly on ice. The nuclei were then sedimented at 2000g for five minutes and lysed by resuspension in 0.2 mM EDTA, 0.2 mM PMSF (Phenylmethyl Sulfonyl Fluoride), pH 7.0 for five minutes at 4\textdegree C. The
suspension was sedimented at 2000g for two minutes and the chromatin was recovered in the supernatant; the yield was estimated by measuring the absorbance at 260 nm (usually 15 to 20 OD units). Digested chromatin and fractionation of the subunit particles was performed as described by FINCH et al. (7). Digestion time with 300 units/ml of micrococcal nuclease was 3 minutes at 37° C and stopped as previously described. 1 ml of 30 to 50 OD of the limited digested chromatin was layered on an isokinetic sucrose gradient and ultra-centrifuged at 27,000 r.p.m. in a SW 27.1 rotor (BECKMAN) for 20 hours.

50 fractions of 20 drops each were collected per tube and the optical density was measured at 260 nm using a BECKMAN 25 spectrophotometer. The recovery of DNA at this level was 70 to 80 percent of the original material. Fractions corresponding to the three upper optical density value of each peak were pooled and extensively dialyzed against sodium chloride 10^{-5}M, 2x10^{-4} M EDTA pH 7.0.

DNA and histone extraction: The compositions of the different fractions were analysed by means of polyacrylamide gel electrophoresis. Dialysed fractions were made 1% SDS, 1M NaCl and extracted twice with a chloroform-isoamyl alcohol (24 : 1) mixture. The aqueous phases were dialysed against water overnight and lyophylised. Samples were then dissolved in Tris-Acetate buffer, pH 7.8, and electrophoresis was performed on 2.5% acrylamide gels as described by LOENING (21). Gels were stained with 2x10^{-6} M Ethidium Bromide.

For histones extraction, the dialysed fractions were made 0.25 N HCl with 1 N HCl under a fast vortex mixing. The precipitated DNA was sedimented at 10,000g, re-extracted with 0.25 N HCl and sedimented again. The combined supernatants were dialysed overnight against distilled water and lyophylised. The dry residues were dissolved in 0.5N acetic acid, 15% sucrose and analyzed by electrophoresis on 15% acrylamide gels, 2.5M urea as described by PANYIM and CHALKLEY (22).

Circular dichroism: C.D. spectra were recorded at 27° C with a CARY 61 spectrometer in a 1 cm light path cell. Concentration of the samples were kept at the maximum value available after dialysis of each pooled fraction (monomer, dimer, and trimer), but no more than 1.2 OD units. The slit was set for an approximate resolution of 1.5 nm, the time constant 10 seconds, the sensitivity was 0.02. The base line was recorded under the same conditions before each spectrum. The zero signal was determined for wave lengths ranging from 350 nm to 330 nm since no detectable absorption occur in this range.
The spectra were calculated, after correction for base line deviation, in terms of ellipticity \((\text{deg} \times \text{cm}^2 \times \text{dmol}^{-1})\) on the basis of DNA residue concentration. Since we were more interested in the qualitative variation of the C.D. of the different subunits, all the spectra were normalized to their maximum ellipticity.

Thermal denaturation curves were recorded on a GILFORD spectrophotometer, modified for melting experiments by WILLIAM RIDGEWAY in this department. Derivative curves were calculated and normalized by using a polynomial curve fitting of degree three for sets of 13 consecutive points. The heating rate was about \(10^{-1} \text{°C/min}\).

Ethidium bromide binding was performed as described in a previous study (23). The total concentration, \(C_e\), of ethidium bromide was calculated for each sample according to its concentration in DNA, in order to maintain the starting DNA/Ethidium ratio constant. This value was taken as 80, and aliquots of this solution were added to a solution of free ethidium bromide of the same concentration. Fluorescence enhancement was measured after each addition, calculations of the bound ethidium bromide, \(C_b\), and the free, \(C_f\), being made as previously described (23). The data are plotted according to the SCATCHARD representation, using \(r = C_b/P\), \(P\) being the DNA concentration in mole of phosphate per liter, for each point of the equilibrium.

RESULTS AND DISCUSSION

A-Fractionation: A typical fractionation pattern is shown in Figure 1. The use of an isokinetic sucrose gradient provides a better resolution than a simple linear gradient. It eliminates the need of a high ionic strength for the fractionation medium as suggested by WOODCOCK and FRADO (24), and preserves the original composition of the extracted subunits. A major part of the non-histone proteins and some of the histones are indeed known to be dissociated in 0.5M NaCl. Increasing the digestion time to 6 min induces the disappearance of the higher order multimers, and increases the relative amount of monomer as compared to the dimer and trimer. However, although we were able to get up to 70 to 80 percent of the fractionated material as monomer, we did not observe the slow sedimenting fragments (3.4S, 5.3S, and 8.6S) described by RILL et. al. (25). For the physical experiments described below, the digestion time was kept constant at 3 min and the nuclease concentration was 300 units per ml for 2 to 3x10^8 nuclei/ml. The three fractions of the highest optical density value from each peak were enough to provide a purity of more than 95 percent for the monomer, 90 percent for the dimer, and 80 percent for the trimer.
The pattern of DNA electrophoresis (figure 2) was found strictly similar to those previously published by other authors ((2) and (7)). In order to analyse the presence of eventual single nicks induced by nuclease digestion in the monomer itself, the DNA extracted from this fraction was heated 10 min in a boiling water bath and chilled quickly on ice. 10 μg of the obtained material were loaded onto a gel and run together with the regular double stranded DNA. A single band was observed, slightly less strained than the corresponding double stranded DNA, but at the same level. No fast moving material could be detected. The digestion was then enough to fractionate the chromatin into subunits and their multimers but not strong enough to remove the 30 base pairs fragment associated with Histone H1 as described by VARSHAVSKY et al. (28).

This result was confirmed by the analysis of histone compositions of the fractions (figure 3) which are found strictly similar for the monomer, dimer, trimer, and native chromatin.

B-Melting curves: Melting curves and their derivatives obtained from fractions prepared according to this procedure are shown in Figure 4. They are essentially monophasic and the melting parameters are given in Table I. Chromatin seems, however, to behave slightly different from the subunits: the melting point is 3°C higher and the transition, as estimated by the
width at half height of the derivative curve, is significantly broader than for the subunits. A monophasic melting profile has been previously described by other workers for the monomer (1)(8), but biphasic or multiphasic profiles have been found for multimers (8) or native chromatin (1). In our preparation of native chromatin the monophasic melting curve that we observe can be transformed in a multiphasic one if the sample is submitted to either shearing or aging (data not shown). A similar effect is obtained with H1.
Figure 4: Normalized melting curves (left) and their derivatives (right) for -o- monomer, -a- dimer, -v- trimer, and -e- native chromatin.

<table>
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<tr>
<th>Sample</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Trimer</th>
<th>Native Chromatin</th>
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<tr>
<td>Tm (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>80</td>
<td>79</td>
<td>82</td>
</tr>
<tr>
<td>Maximum Ellipticity (θ)</td>
<td>1.4 ± 0.3 x 10^3</td>
<td>1.3 ± 0.2 x 10^3</td>
<td>1.3 ± 0.2 x 10^3</td>
<td>1.9 ± 0.1 x 10^3</td>
</tr>
<tr>
<td>θ275/θ282</td>
<td>0.32</td>
<td>0.56</td>
<td>0.66</td>
<td>1</td>
</tr>
<tr>
<td>KN</td>
<td>1.1 x 10^5</td>
<td>1.4 x 10^5</td>
<td>1.9 x 10^5</td>
<td>2.5 x 10^5</td>
</tr>
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Table 1: Physico-Chemical Properties of Chromatin Subunits as compared to Chromatin.
depleted chromatin (23). The presence of a monophasic pattern seems then to be very sensitive to any denaturating conditions, the result of which is a multiphasic melting pattern. Our pattern of monophasic melting curve is obviously different from those obtained by WOODCOCK and FRADO (14) with subunits prepared in high ionic strength. From their experiments these authors proposed a strongly heterogeneous model for DNA organization in the chromatin fiber. Our result can more likely be interpreted in terms of a highly and homogeneously stabilized structure, as far as native state of chromatin is concerned. However, stabilization of DNA in chromatin against thermal denaturation reflects only an electrostatic screening of the protein components on the phosphate backbone, at least in the low resolution conditions used in this experiment. It is well known that a similar effect can be obtained on DNA alone by simply increasing the ionic strength of the solvent. Our melting curves, then, refer only to such an effect but do not provide further evidence on the conformation of DNA in particles as compared to chromatin. Such evidence, however, is described in the following experiments.

C-Circular Dichroism: Figure 5 shows the normalized positive band of the CD spectra from monomer, dimer, trimer, and chromatin. Their shapes are obviously different: CD of monomer is characterized by a maximum ellipticity at 282 nm and a light negative signal at 300 nm. The dimer has an increased minimum at 300 nm, and the maximum is now shifted to 280 nm, probably because of the appearance of a shoulder at 275 nm. For the trimer, this effect is reinforced and the shoulder is more pronounced. The maximum ratio $\theta_{275}/\theta_{282}$ is finally reached for native chromatin. In this last sample, however, the negative ellipticity at 300 nm is reduced and becomes comparable to that of monomer. Table 1 shows the calculated ellipticities before the normalization. The whole positive peak of chromatin is markedly enhanced as compared to that of any other fraction.

A similar result has been described previously by SAHASRABUDDHE and VAN HOLDE (1) when comparing the CD properties of their P S particles with that of chromatin. However, the CD spectrum of the P S particles showed a significant shoulder at 275 nm that we did not observe in our monomer fraction. This discrepancy can probably be attributed to the difference in the mode of preparation of the monomer and the P S particles. In this later case, a precipitation method was used which cannot distinguish between the elementary particles and the higher order multimers.

A rigorous interpretation of circular dichroism spectra of nucleoproteic
systems is presently not available, but some particular features from certain chromosomal components are, however, well established. Histones, first, have no CD signal in the 260 - 330 nm range but they are known to induce a drastic decrease of the ellipticity of DNA at 280 nm (26)(35)(36)(37).

From Figure 3 and from a quantitative estimation of the ratio $H_1/H_3$ by densitometric scanning of the electrophoretic gels, it appears that the histone composition of monomer, dimer, and trimer are identical to that of native chromatin. Namely histone $H_1$ is still present in the monomer: the mild digestion conditions used in these experiments and the selection of the three highest optical density fractions in the monomer peak avoid the heterogeneous composition of monomer which has been described by VARSHAVSKY et al. (28). It seems, then, unlikely that the differences in the CD behavior that we observed can be attributed to changes in histone composition.

Non-histone proteins have also been found to have a signal contribution in the positive CD band of chromatin (29)(30) but it is not yet clear whether this effect is due to a direct contribution or a result of their
interaction with DNA. Since no detailed work on the non-histone composition of the subunits has been published, their possible contribution to the CD properties that we observe cannot be discarded a priori.

D-Ethidium Bromide Binding: Figure 6 shows the binding isotherms of ethidium bromide to monomer, dimer, and trimer. Such binding isotherms, which are characterized by their convex shape, have been previously found to occur in native chromatin (17). They can be analyzed in terms of a binding occurring on two sets of independent binding sites differing by their binding constants. In the case of native chromatin, each set of binding sites was found to follow the excluded site model mechanism proposed by CROTHERS (31). The first class has a very high binding constant and represents 13 percent of the total DNA; the second class has a binding constant consistent with the screening effect expected from electrostatic interaction of histones with the DNA phosphate backbone; it represents 82 percent of the DNA. (For a detailed description of these properties, see reference 32)).

![Figure 6: Binding isotherms of ethidium bromide to the different fractions (same symbols as in Figure 4) in a SCATCHARD'S representation; the total concentration $C_t$ is expressed in mole/liter.](image-url)
These two binding site processes are still present in the chromatin subunits, including the monomer. However, the convexity of the isotherms increases as the number of subunits increases. This means that the difference between the binding parameters of each class of binding sites is deepened. Such an assertion can be reinforced by a more quantitative estimation of the binding parameters of the primary binding sites (high affinity) which occur almost independently for the smaller values of \( r \). Indeed, if we consider only this part of the isotherm, and we make no assumption on the binding mechanism, then the well-known equation:

\[
\frac{r}{C} = K (N-r)
\]

where:

- \( r \) is the amount of bound dye per phosphorous molecule, \( (C_b/P) \),
- \( C \) the free dye concentration at the equilibrium, \( (C = C_t - C_b) \),
- \( C_t \) the total concentration of dye used for a binding experiment,
- \( K \) and \( N \) are the apparent binding constant and the number of binding sites respectively,

This equation can be applied.

For very low values for \( r \), i.e. for very high values of \( P \), then \( r \ll N \) and this equation can be transformed into:

\[
\frac{C_b}{P (C_t - C_b)} = KN
\]

or this can be arranged as:

\[
\frac{C_b}{C_t} = 1 - \frac{1}{KN} x \frac{1}{P}
\]

A plot of \( C_b/C_t \) versus \( 1/P \), for high values of \( P \), must be a straight line, the slope of which is \(-1/KN\). We can then estimate the variation of \( KN \), the product of the apparent parameters of the high affinity binding sites, very simply from such a representation.

Figure 7 shows that this approximation is valid, at least for the lowest \( r \) values as it was assumed. Table I gives the values of the product \( KN \) calculated from this representation. This parameter, again, increases from the monomer to the trimer, and its maximum value is reached for native chromatin. At this level, it does not seem essential to known which is affected, \( K \) or \( N \), by chromatin fractionation to give a lower value of the product, but it is more interesting to consider the extent of the variation. Figure 8 shows the variation of \( KN \) as a function of the number of subunits (\( n \)) in the multimer. The value of native chromatin is indicated by the horizontal
Figure 7: Proportion of bound ethidium bromide versus the reciprocal of the DNA concentration expressed in mole of phosphorus per liter. The slope of the obtained curves are $-1/KN$, $K$ and $N$ being the apparent binding parameters of the high affinity binding sites.

The dotted line. A straight line drawn through the three points intercepts the dotted line at the level of $n=5$. In fact, it seems unlikely that the variation of $KN$ as a function of $n$ would be linear over the whole range: $KN$ would probably follow an asymptotic law to reach the final chromatin value.

However, the value of five subunits, probably more, gives an estimation of the minimum number of subunits required to form "an equivalent native entity".

Since the presence of high affinity binding sites in chromatin has been attributed to a constrained state of the DNA upon histone interaction (32), it is interesting to notice that probably more than five subunits are needed to maintain this particular state. The fact that we observe a continuous increase of the $KN$ product when the number of subunits increases, suggests that such constraints are the result of a long range effect of the subunits interaction. Whether such interactions occur in a linear arrangement or in a folded structure of the subunits is still to be determined, and so is the nature of the components which maintain this structure.
CONCLUSION

The present study dealt with some properties of DNA in chromatin subunits obtained by fractionation of native chromatin after mild nuclease digestion. A particular emphasis was made on the effect of the sizes of the different fractions (monomer, dimer, trimer) when compared with native chromatin.

Melting experiments, first were unable to reveal strong heterogeneity in the mode of binding of proteins to DNA. They lead to the conclusion that the electrostatic stabilization of the DNA duplex is strong all along the chromatin fiber, although the broadness of the transition suggests that some heterogeneity in the mode of interaction of proteins with DNA can occur in the chromatin fiber as well as in subunits. Such differential stabilizations are found to take place in model systems, such as Histone - DNA reconstituted complexes (33). This result does not support a model of chromatin consisting of fragments of DNA where the proteins are loosely bound alternating with fragments where they are strongly bound. It is more consistent with a model of chromatin consisting of subunits closely bound to one another, leading to a rather continuous fiber structure, as has been shown by several electron microscopic studies (34)(6).

Nevertheless, conformational heterogeneity of DNA in native chromatin found by means of ethidium bromide binding studies (17), (32), was found
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again in chromatin subunits, including the monomer (although with some kind of attenuation). This heterogeneity was associated with the appearance of a shoulder at 275 nm in the positive band of the circular dichroism spectra in addition to the maximum peak at 282 nm. The ratio $\theta_{275}/\theta_{282}$ increases from the monomer to trimer and reaches a maximum in native chromatin.

A similar behavior was found for the product KN of the apparent binding parameters of the high affinity binding sites of chromatin. The study of the variation of this product with the sizes of the fractions, suggests that a minimum number of subunits is required to confer to chromatin its native properties. It appears that this number cannot be less than 5.

If we assume that the properties found in this study have some kind of correlation with the functional properties of chromatin - indeed, we have previously suggested (32) that the binding mechanism of ethidium bromide to chromatin might reflect analogous properties for other ligands, whether they are small molecules of pharmacological interest, or macromolecules implicated in the expression of chromatin functions - then our general conclusion would be that the native state of chromatin requires a particular arrangement of the monomers together, leading to the idea of the existence of a functional subunit in chromatin, made up of a minimum number of structural subunits. We postulate that the integral specific properties of chromatin can arise only from such functional subunits.

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