Isolation and physical characterization of a stable core particle

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ABSTRACT

Core particles were prepared from mature chicken erythro-
cytes chromatin, according to the method of Lutter (J. Mol. Biol.
124, 391, 1978) with one major modification : after the second
digestion, zonal centrifugation was used to isolate the core
particle, instead of chromatography on Sepharose 6B.
By using circular dichroism and electron microscopy, we were
able to follow each step of the preparation and to offer an
explanation of the discrepancies found in previous preparations
and in our own preparations.

INTRODUCTION

The core particle designates the specific assembly of a
short piece of double stranded DNA (145 base pairs) to which are
bound eight molecules of histones H3 H4 H2A H2B with the exact
stoichiometry of two copies per each histone molecule. Further-
more, histones are assembled in an octamer where specific inter-
actions between histone molecules insured a given but unknown
geometry of the complex. DNA is wrapped around the core histone
but so far, only the preliminary outlines of the precise tertia-
ry structure were determined (1). The core particle appears the-
refore as a kind of common structural subunit of the chromatin
which is defined in terms of a given molecular complex rather
than in terms of a well-specified functional unit. This explains
the discrepancies found in the literature between the different
physical properties of the core particle, because of the diffi-
culty to prepare and isolate a well-defined and reproducible
material. For example, a general agreement was obtained relative
to M and S values of the core particle, but large differences

were present when circular dichroism spectra or melting profiles
are concerned (2-8).

In this work, by using concurrently circular dichroism and
electron microscopy, we were able to follow carefully each step
of the preparation, to obtain a well-characterized, stable core
particle from chicken erythrocyte chromatin and to offer an ex-
planation of the different types of discrepancies which are
found between different samples obtained with a given method of
preparation, including that one used in this work.

METHODS

Core particle preparation

The method used was essentially that described by Lutter
(9) for rat liver : nuclei were prepared from mature chicken
erythrocytes according to the procedure of Hewish and Burgoyne
(10) modified as previously described (11) and adjusted to 50
A_{260} units/ml. Nuclei were then digested with 150 units/ml mi-
crococcal nuclease (Worthington), during 30 sec. at 37°C, relea-
sing about 1 to 2% acido soluble. Digestion was stopped by addi-
tion of EDTA to a final concentration of 2 mM. The lysis of nu-
clei was achieved by suspending the pellet in 0.2 mM EDTA pH 7.9,
giving rise to 70-80% soluble long chain chromatin. H1, H5 as
well as non-histone proteins were then removed from this chroma-
tin by addition of salt up to 0.65 M NaCl (instead of 0.45 M as
used for rat liver). The sample was then applied to a 5 x 100 cm
column of Sepharose 4B (9). Pooled fractions containing the strip-
ped long chromatin were dialysed against 5 mM Tris HCl (pH 7.5),
20 mM ammonium acetate, 0.2 mM EDTA, 2 mM-2-mercaptoethanol and
concentrated to 30-50 A_{260} units/ml.
This concentrated partially stripped chromatin was redigested
with micrococcal nuclease (90-150 units/ml). Samples were remo-
ved at different times, as indicated by Lutter (9) and the DNA
was analysed to determine at what time all DNA species have
disappeared except the major band corresponding to the 145 base
pairs material. Core particles were isolated by zonal centrifu-
gation (and not by chromatography on Sepharose 6B), for 21 h at
38000 RMP (Rotor Beckman Ti XIV), in a 5-30% sucrose gradient,

in presence of 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7. The pooled core particles were dialysed against 1 mM phosphate buffer pH 7.4, 5 mM NaCl, 1 mM EDTA, 0.1 mM PMSF and concentrated through an Amicon XM-50 membrane. The preparations were stored at 4°C in the presence of sodium azide.

Histones were resolved by electrophoresis on SDS gels according to the method of Weintraub et al. (12). DNA was extracted from the chromatin or the core particles by treatment with proteinase K and the phenol-chloroform-isoamyl alcohol mixture and precipitated finally with ethanol. The length of DNA was measured in polyacrylamide gels according to Peacock and Dingman (13), using Hae III restriction enzyme fragments of PM_2 for calibration ; sizes are taken from Tatchel and Van Holde (5).

Circular dichroism measurements (CD)

All the experiments were performed in the same medium : 1 mM phosphate buffer pH 7.4, 5 mM NaCl, 1 mM EDTA.
Circular dichroism spectra were recorded at 20°C with a Roussel Jouan DC III (Jobin-Yvon) in a cylindrical cuvette with 1 cm optical pathlength. DNA concentration was about $1.5\ 10^{-4}$ M (P).
In each case the turbidity of solutions, as controlled by light scattering at 320 nm, was negligible.
The results are presented in terms of molar ellipticity $|\theta|$ (in degree, cm^2, decimole^{-1}), based on the molar nucleotide concentration determined from an extinction coefficient of 6.600.

Electron microscopy

The samples used for CD measurements were fixed in 0.1% glutaraldehyde, then diluted to 0.5 µg/ml in 5 mM Triethanolamine, 5 mM NaCl, 0.2 mM EDTA, 0.1% glutaraldehyde, pH 7.4 and spread on positively charged carbon coated grids according to the method of Dubochet et al. (14) as previously described (15).

Sedimentation constant and equilibrium

Sedimentation velocity and sedimentation equilibrium measurements were performed in a model E Spinco ultracentrifuge equipped with a digital scanner. Absorbances are measured within

$\pm$ 1 x 10^{-3} unit and distances from the axis are determined within $\pm$ 4 μm.

All of the measurements were conducted in 20 mM NaCl, 10 mM Tris buffer pH 7.4, 0.1 mM EDTA.

RESULTS

PREPARATION

In figure 1, a typical separation of core particle by zonal centrifugation is given, as well as the electrophoresis of the corresponding core DNA and histones. The resulting core particle contains two of each of the histones H2A, H2B, H3, H4 and a piece of DNA of 145 $\pm$ 3 b.p. (see ref. 15, fig. 1).

CHARACTERIZATION

Since we have defined the core particle as a structural entity, its characterization must rely on rather physical than biochemical measurements.

Sedimentation data

The core particles which have been prepared as described above have a $s_{20,w}$ = 10.90 $\pm$ 0.03 S, in 20 mM NaCl, 10 mM Tris buffer, pH 7.4.

Equilibrium sedimentation was realized with short columns of solution (< 0.3 cm) at 4°C and 9.800 RPM. The plots of Log A_{260} versus r^2 were straight lines with regression coefficients close to 0.999. The true molecular weight was obtained by studying three solutions with absorbances of 0.337, 0.224 and 0.168 respectively and by extrapolation to c = 0 of the reciprocal of the apparent molecular weights determined for each concentration with a value of the partial specific volume $\bar{v}$ = 0.661 (16) and a specific mass of the solvent equal to 1.0016, the extrapolated molecular weight was found to be M_W = 196 $\pm$ 4 x 10^3. This value is in good agreement with that expected (198.5 x 10^3) from an assembly of 145 bp of DNA (89,900) and an octamer of histones (108,600).

Circular dichroism

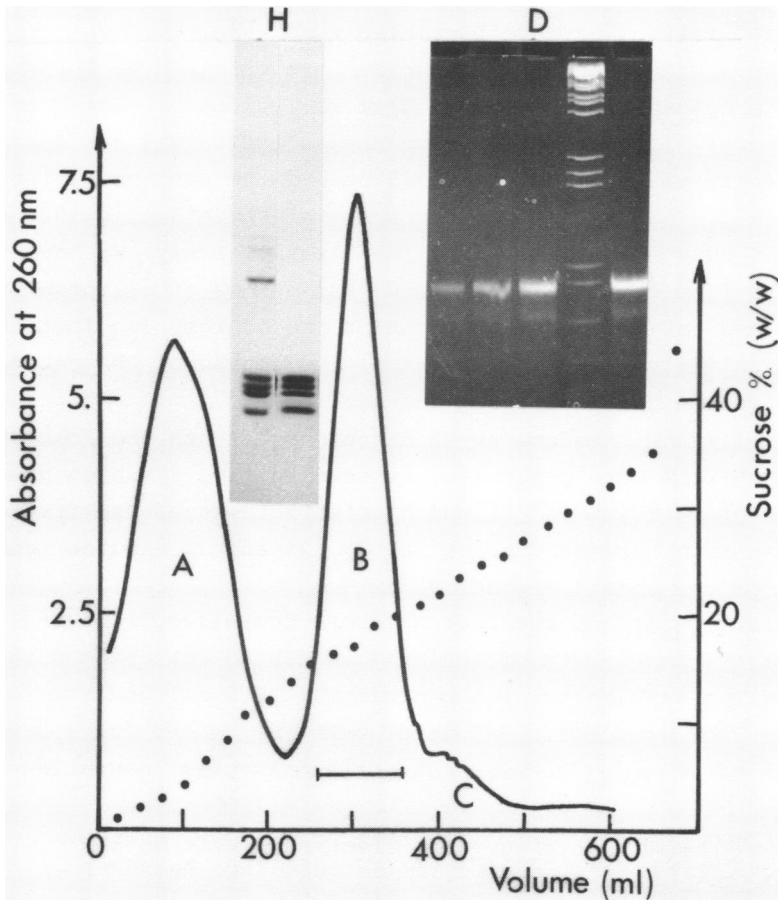CD spectra were used only in the range 260-320 nm in

Figure 1 - Large scale sucrose gradient zonal centrifugation of partially stripped chromatin redigested with micrococcal nuclease.
(———) absorbance at 260 nm   (•••••) sucrose concentration
A. Acido soluble material   B. Core particle
C. Compact dimer
The inset (D) shows the electrophoresis of core DNA in 6% polyacrylamide (see Methods). Channels from left to right contain
(1) 0.1 μg ; (2) 0.2 μg ; (3) 0.4 μg ; (5) 0.6 μg DNA ; (4) Hae III restriction fragments of PM$_2$ DNA.
The inset (H) shows the electrophoresis of the histones (see Methods). Channels contain (1) Total histones of erythrocytes (as reference) ; (2) Histones of core particle.

which the contribution of proteins is negligible. Ten diffe-
rent preparations of core particles were made.
The general features of the spectrum are retained in these dif-
ferent preparations but substantial variations are detected
(not shown). If, for example, we use as indicators the maximum
ellipticity value $|\theta|_{max}$ at 283.5 nm and the minimum ellipticity
value $|\theta|_{min}$ at 294.5 nm, their range of variation is 1000 <
$|\theta|_{max}$ < 2000 and - 590 < $|\theta|_{min}$ < 0 respectively. We have thus
tried to find correlations between variations of ellipticity
values and variations of preparation procedures. Two factors
were peculiarly investigated : the time course of the second
digestion and the last step of isolation :

(i) the time course of digestion affects the homogenei-
ty of DNA from core particles, as seen in fig. 2, which gives
for one preparation (n° X) the electrophoresis of DNA fragments
obtained from chromatin after 15, 20, 25 and 30 minutes respec-
tively of the second digestion process.
The ellipticities of the four aliquots decreased from 2160 to
1490 at the wavelength of the maximum (283.5 nm), from 0 to
-280 at the wavelength of the minimum (294.5 nm) and from 660 to
0 at 270 nm. The lowest values of both $|\theta|_{max}$ and $|\theta|_{min}$, as well
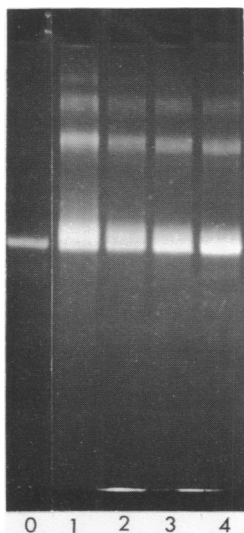as that of $|\theta|_{270nm}$ corresponds to the narrowest migration band

Figure 2 - Electrophoresis on 6%
polyacrylamide gels (see Methods)
of the DNA extracted from (H1,H5)-
stripped chromatin and redigested
with micrococcal nuclease for the
times indicated : channels from
left to right were 15, 20, 25 and
30 min. The marker is DNA from
purified core particle (Channel 0)

of core DNA, i.e. for a 30 minutes digestion time (figure 2).

    (ii) The chromatography on Sepharose 6B in 0.1 M NaCl
as the last step of core particle isolation was compared to the
zonal centrifugation procedure. For the same digestion time
(30 minutes), the θ values are higher after chromatography than
after zonal centrifugation, and correspond to the upper limits
of the variation range given above.

Electron microscopy

    Like circular dichroism, electron microscopy reveals
differences between preparations which are illustrated in figure
3. In each preparation a given amount of core particles appear
under the electron microscope as partially unfolded particles
presenting a "tail" of variable length, corresponding likely to
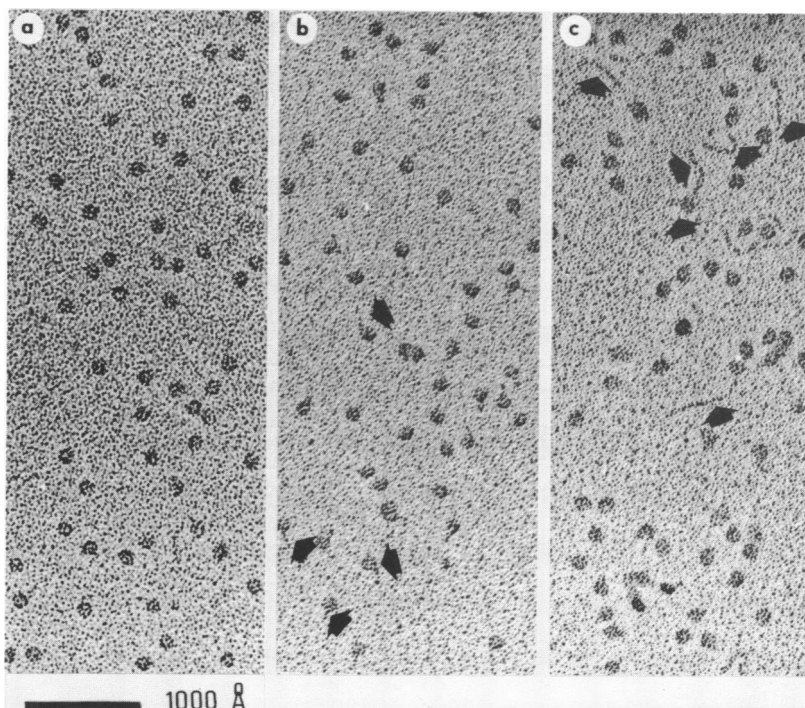naked DNA. Core particles were thus arbitrarily divided into two

Figure 3 - Electron microscopy of core particles preparations.
a) and b) Preparations n° VI and X respectively.
c) Preparation n° IX after chromatography on Sepharose 6B.

subpopulations of unfolded (with tail) and native (without tail) particles.

The relative estimation of each of these subpopulations was made only on five preparations (VI, IX, X, XI, IX*) after counting about 1000 particles and is given in table I.

It appears clearly (i) a correlation between the percentage of unfolded particles and the ellipticities values. The lower the percentage of "tails", the lower the CD signal ; (ii) an influence of the chromatographic isolation in 0.1 M NaCl upon the unfolding of core particles.

Stability

In view of the correlation found previously between ellipticity values and morphologies under electron microscope, CD measurements were used to follow the stability of core particles when storage conditions or solvent medium were modified. In the first case, the results are given in table II.

If storage at low ionic strength is made during 7 weeks, it is better either to keep the solution in the cold room, or to freeze it in presence of 50% glycerol. When freezing is made without glycerol, ellipticities values are significantly higher. Moreover, the storage in phosphate buffer has to be prefered to the storage in Tris buffer.

Table I - Core particle preparations : Correlation between CD spectra and electron microscopy.

Preparation n°	$\|\theta\|$ degree, $cm^2 dmole^{-1}$ ($\pm$ 100) at λ (nm)			Percentage of core particles with "tails" as obtained by electron microscopy
	283.5 Max.	294.5 Min.	270	
VI	1030	− 590	− 590	2
IX	1470	− 310	− 400	4
X	1490	− 280	0	6
XI	1240	− 390	− 390	6
IX(*)	2020	0	230	13

(*) After chromatography on Sepharose 6B

Table II - Core particle preparations ; influence of the solvent and way of storage

Solvent and storage conditions	$|\theta|\,degree,cm^2dmole^{-1}$	
	Maximum	Minimum
Fresh preparation in buffer (A)	1240	- 390
After 7 weeks at 2-4°C	1270	- 420
After 7 weeks at -20°C buffer (A) + 50% glycerol	1130	- 420
After 7 weeks at -20°C without glycerol	1990	- 210
Fresh preparation in buffer (A) + 0.1 M NaCl	1160	- 370
The same, after 16 days	1300	- 320
The same, after 23 days	1480	- 320

Buffer (A) is 1 mM phosphate buffer pH 7,4, 5 mM NaCl, 1 mM EDTA

In the second case, the effect of increasing ionic strength on the stability of core particle was followed by CD measurement. The change of $|\theta|_{280}$ with ionic strength is represented in figure 4, by a sigmoidal curve indicative of a change of state. When the ionic strength is increased from 0.25 M to 2 M and then decreased to come back to its initial value, the molar ellipticity is exactly recovered provided the core particle concentration is maintained high enough all along the process (unpublished experiments).

DISCUSSION

The preparation of core particles, their physical characterization and their stability during the storage or solvent medium modifications were studied respectively.As it was already shown in previous papers (15,17), the combination of CD spectroscopy and electron microscopy allows us to describe the core
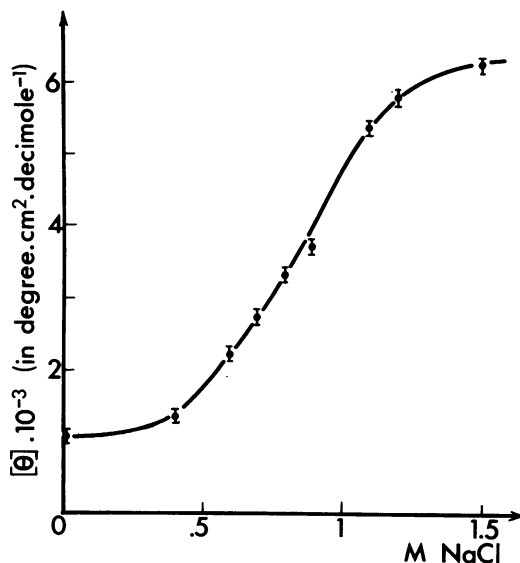
Figure 4 - Effect of increasing ionic strength on the stability of core particle followed by circular dichroism.

particle at two levels of organization : the conformation of DNA and the morphological features of the subunit. The good correlation between the results obtained with the two methods lead us to precise as well as to discuss the meaning of the physical parameters which were used to characterize the chromatin subunits.

At first, it is tempting to consider the ellipticities values as indicative of the native state of the core particle. This one would be the "best" one when the CD value is the lowest. This assumption was true for chromatin (17). Such a simple criterion appears indeed valid when one considers the influence of either the DNA heterogeneity or the DNA unfolding. In the first case (digestion time lower than 30 minutes in our conditions), the presence of DNA fragments longer than 145 bp raises the values of the CD signal. In the second case, the occurrence in some particles of partially naked DNA increases again the ellipticities values. As shown clearly in figure 3 and table I, the lowest CD signal corresponds to only 2% of core particles with a "tail". In other words, and according to the criterion

exposed above, the preparation n° VI has to be considered as the best one we were able to realize. We have however to point out that if we agree to decide of the quality of a core particle preparation from the ellipticities values in the range 260 - 320 nm, we are so far unable to master all of the parameters during the isolation procedure in order to obtain in any case the lowest values of ellipticities, i.e. the "best" core particle. The percentage of partially unfolded particles cannot be predicted and moreover we have been unable to prepare core particles without a few percent of them with "tails".

The difference in stability of core particles, when submitted to external stresses like change of ionic strength (18, 19), column chromatography, binding of ethidium bromide (15) or increase of temperature (20), is reflecting the initial heterogeneity of core particles population. The presence of a small percentage of acetylated or phosphorylated histones or of variant of histones (21) could explain such a behaviour and we are now trying to examine carefully chemical modifications of histones among the different classes of core particles.

As a conclusion, we have to emphasize the effect of partially unfolded DNA (presence of "tails" under the electron microscope) on other properties of core particles :

a) in our case the melting profile of core particles is always monophasic, with a T_m close to 82°C in 10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA. However, in presence of 13% of partially unfolded core particles as found in preparation n° IX after column chromatography (see table I), a biphasic profile is taking place. This means the presence of a secondary melting process taking place before 82°C and corresponding to the small amount of naked DNA.

b) In a recent study of transcriptional activity of core particles in presence of RNA polymerase II (22), the transcription was found to occur only on partially or totally unfolded DNA.

The simultaneous use of electron microscopy and CD measurement, in order to characterize a stable and well-defined core particle appear as decisive a tool as in our previous studies of chromatin (17), ethidium bromide binding (15) or

influence of temperature (20).

The biochemical and now classical characterization of chromatin material (digestion pattern of nucleases) appear therefore as only necessary but not sufficient to detect fine changes in conformation.

If any material (core particle, oligonucleosome, chromatin) was submitted to physical testing before to be used, many discrepancies present in the literature would disappear.

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