Biophysical Chemistry 14 (1981) 123–133
Elsevier/North-Holland Biomedical Press

CORE PARTICLE STABILITY CRITICALLY DEPENDS UPON A SMALL NUMBER OF TERMINAL NUCLEOTIDES

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Received 28 January 1981

An apparently homogeneous population of core particles is in fact composed of three subpopulations which behave differently when exposed to a high concentration of ethidium bromide or to 0.6 M NaCl. These subspecies have been identified by the use of several techniques, viz., electron microscopy, sedimentation velocity and circular dichroism. The electrophoretic analysis of their DNA leads to the conclusion that core particle stability critically depends upon a small number of terminal nucleotides.

1. Introduction

It seems well established now [1,2] that most of the core particle DNA is largely accessible and recent work [3] has suggested that the actual number of histone-DNA phosphate contacts is probably much lower than that previously supposed. In this study, we show that the ends of core particle DNA are stabilized by a very small number of strong ionic interactions. There is a critical length for core particle DNA, for which at least one of the two terminal 'blocking' zones is absent. Consequently, a so-called 'standard' population of core particles, which one generally considers as homogeneous, is in fact composed of three different subspecies according to their behaviour in response to destabilizing processes such as ethidium bromide intercalation or increase in ionic strength.

2. Material and methods

The so-called 'buffer R' is composed of 20 mM Tris pH 8,1 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF).

2.1. Core particle preparation and characterization

Chicken erythrocyte core particles were prepared according to Lutter's method [4] modified as described previously [5]. The main steps of the preparation are the depletion of H1, H5 and non-histone proteins from long-chain chromatin by chromatography on Sepharose 4B in a medium containing 0.6 M NaCl, and the digestion of this resultant chromatin until 30% acid-soluble material is obtained. Then the separation of core particles was performed by zonal centrifugation and the particles were characterized as described before [5].

The most important features of such core particles are the absence of non-histone proteins (because of the treatment of the chromatin in 0.6 M NaCl), the absence of 160 base-pair species and the presence of 'flush ends' on core particle DNA (because of the extensive redigestion of the chromatin deprived of H1 and H5 [6]).

2.2. Electron microscopy

To a solution of core particles in buffer R at a concentration of 1.5×10^{-4} M (expressed in phosphate concentration) was added a solution of ethidium bromide in buffer R, so as to obtain a dye/phosphate ratio of 0.2. The resulting solution was diluted to 0.5 μg/ml (expressed in DNA concentration) with a buffer containing 10 mM triethanolamine (TEA), pH 8, and 0.2 mM EDTA, either directly or after fixation with 0.1% glutaraldehyde.

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A 50 μl drop of solution was deposited on a carbon-coated grid, positively charged according to Dubochet's technique [7] and left for adsorption during 5 min.

A solution of core particles in buffer R at a concentration of 50 μg/ml (expressed in DNA concentration) was dialysed for 24 h against buffer R + 0.6 M NaCl. The resulting solution was diluted to 0.5 μg/ml with a buffer containing 0.6 M NaCl, 10 mM TEA, pH 8, and 0.2 mM EDTA, either directly or after fixation with 0.1% glutaraldehyde. A 50 μl drop of solution was deposited on a carbon-coated grid, positively charged according to Dubochet's technique [7] and left for adsorption during 5 min.

After adsorption on the grids, the samples were stained with 1% uranyl acetate for 1 min, then rinsed with distilled water, air dried and rotatively shadowed with platinum under an angle of 8°.

The grids were examined in a Siemens 101 Elmiskop.

2.3. Sucrose gradient sedimentation

Fractions of a solution containing core particles at a concentration of 3.75×10^{-3} M phosphate with ethidium bromide at dye/phosphate ratio of 0.2 were layered on the tops of tubes containing 5–20% isokinetic sucrose gradients. Sedimentation was performed with an SW 41 rotor at 38000 rpm for 16 h.

2.4. Analytical sedimentation

The sedimentation studies were carried out in a Spinco E Analytical Centrifuge equipped with a digital scanner [8] and an electronic digital speed control. Absorbances are measured within $\pm 3 \times 10^{-3}$ units in velocity measurements. The distances from the axis are determined within ± 4 μm. The period of rotation is within ± 1 μs.

The measurements of velocity in boundary sedimentation are done in the nominal solvent: buffer R or buffer R + 0.6 M NaCl. For the band sedimentation, the bulk solvents are buffer R + 0.1 M NaCl for the solutions in buffer R, or buffer R + 0.6 M NaCl + 5% sucrose for the solutions in buffer R + 0.6 M NaCl. These concentrations of the bulk solutions are sufficient to ensure the stability of the moving band. The density and the relative viscosity of the bulk solvents are determined for each experiment to allow corrections to the standard conditions.

In any case, absorption measurements allow us to operate in a range of concentration low enough to assume with good accuracy that the experimental values of s and D were relative to zero concentration (see for example table 1). Moreover, in 0.6 M NaCl, the efficient screening of charged core particles made the corrections due to the primary charge effect negligible.

The data of band sedimentation were processed according to Vinograd et al. [9,17]. With the data obtained from the leading half of the fast band, it was possible to determine the diffusion coefficient of the nucleosome in 0.6 M NaCl and to calculate the reduced second moment of mass Σ_2. In order to do that, the band sedimentation pattern of a hypothetical mixture containing 10% of free DNA ($s = 5.2$ S, $D = 3.25 \times 10^{-7}$ cm$^2 \cdot$ s^{-1}) and 90% of native core particle ($s = 10.8$ S, $D = 3.9 \times 10^{-7}$ cm$^2 \cdot$ s^{-1}) has been simulated with the aid of a program developed by Claverie et al. [10]. By introducing the experimental conditions corresponding to a real sedimentation run, it was possible to determine with good accuracy the time beyond which the leading half of the fast band is no longer perturbed by the presence of the slow component.

2.5. Circular dichroism

Measurements were made with a Mark III Jobin Yvon dichrograph. We used a cylindrical cuvette with 1 cm optical pathlength. Solutions of core particles in buffer R or in buffer R + 0.6 M NaCl had an optical density of 1 at 260 nm.

2.6. DNA electrophoresis

The length of DNA fragments was measured in 10% polyacrylamide slab gels (40 cm long) according to Peacock and Dingman [11] using Hinf I restriction fragments of Cauliflower Mosaic Virus for calibration [12]. The gels were run for 18 h at 300 V, stained with ethidium bromide (1 μg/ml)

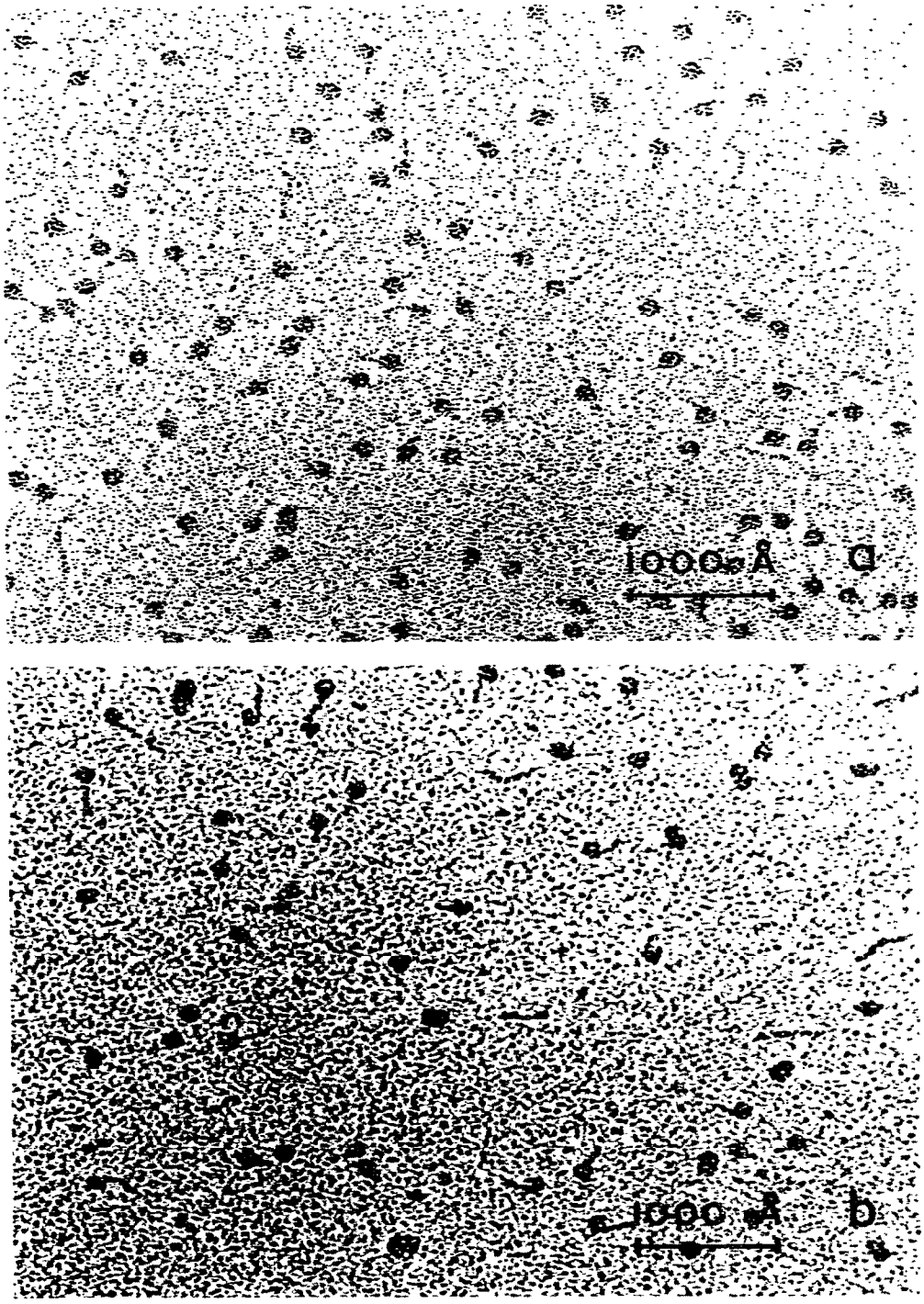

Fig. 1. Electron micrographs of core particles (a) at low ionic strength after ethidium bromide intercalation; (b) in 0.6 M NaCl. Experimental conditions are described in details in section 2.2. Efficiency of adsorption is lower in 0.6 M than at low ionic strength (G. de Murcia and T. Koller, submitted).

and photographed under ultraviolet illumination.

The DNA lengths were determined from scans of the negatives with a TRANSIDYNE 2955 scanning densitometer.

3. Results and discussion

3.1. Heterogeneous behaviour of an apparently homogeneous population of core particles in response to destabilizing agents

3.1.1. Core particle destabilization induced by ethidium bromide intercalation

We have previously shown by electron microscopy [13] that, in the presence of ethidium bromide at a high dye/phosphate ratio (>0.2), half of the core particles remain correctly folded while the other half unfold. Most of the time this unfolding is partial and polar, but sometimes it is total (fig. 1a). The proportion of these particles which completely dissociate into DNA and histones represents about 10% of the population as estimated by counting of a thousand particles. When the whole population of ethidium bromide-modified core particles is layered on a 5–20% isokinetic sucrose gradient at low ionic strength, one obtains two peaks (fig. 2). As shown by electron microscopy, peak 1 (which does represent 10% of the total DNA) is exclusively composed of naked DNA, and peak 2 is composed of a mixture of both folded and partially unfolded core particles. We get the same pictures with or without glutaraldehyde fixation. The electrophoresis of the histones extracted from peak 2 core particles shows a correct stoichiometry of the four histones, indicating that there is no release of H2A or H2B from the partially destabilized core particles.

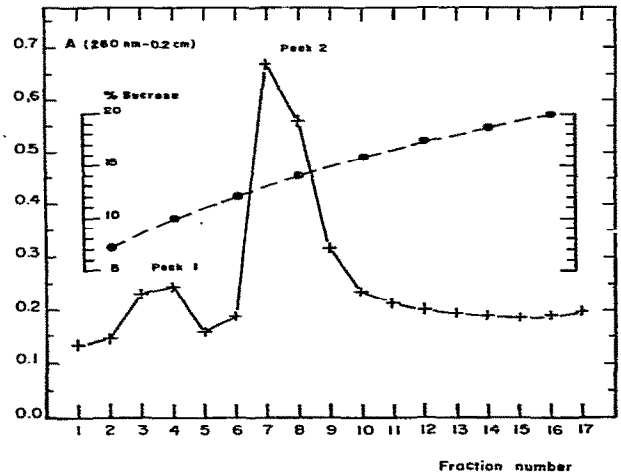

Fig. 2. Profile of separation obtained with the 5–20% isokinetic sucrose gradient of core particles perturbated by ethidium bromide (see section 2.3.). Fractions of 0.5 ml were collected from the top to the bottom of the tubes.

3.1.2. Core particle destabilization induced by ionic strength increase (0.6 M NaCl)

3.1.2.1. Electron microscopy. Quite similar results (fig. 1b) are obtained when core particles are placed in 0.6 M NaCl and observed at this ionic strength under the electron microscope. Here again counting of several thousands of particles reveals the presence of 50% apparently intact core particles, 40% partially unfolded particles and 10% naked DNA, the percentage being given with an uncertainty of 2%. We also get the same pictures with or without glutaraldehyde. It means that, as previously mentioned in the case of ethidium bromide action, an apparently homogeneous population of core particles is in fact composed of three subpopulations which behave differently in terms of their respective stability when exposed to 0.6 M NaCl. The first one is very stable, the second less stable and the third completely unstable. By the other techniques used (circular dichroism and sedimentation) it is impossible to discriminate between the most stable subpopulation and the partially destabilized one, i.e., they are detected together in only one set, the third unstable subpopulation appearing as naked DNA.

3.1.2.2. Circular dichroism. The molar ellipticity $|\theta|$ at 283.5 nm is 2000 degree $\cdot$ cm^2 $\cdot$ dmol^{-1} instead of 1250 for core particles at low ionic strength. It can be explained by the contribution of 10% naked DNA and 90% core particles in which DNA has a proper folding, i.e., if not exactly the same, at least very close to that at low ionic strength.

Table 1
Boundary sedimentation of solutions of core particles at different initial concentrations in buffer R + 0.6 M NaCl: values of s for the major component.

C (O.D. 260 nm, 1 cm)	s (S)
3.45	9.57 ± 0.15
0.80	9.41 ± 0.15
0.54	9.38 ± 0.15
0.27	9.59 ± 0.15

3.1.2.3. Analytical centrifugation. (a) The *boundary sedimentation* was carried out at different initial concentrations. At all concentrations, the boundary exhibits two transitions. The major one gives s values listed in table 1. For the highest concentration, the minor transition has $s_{20,w} = 4.80 \pm 0.15$ (fig. 3). The amount of this small component, corrected for the radial dilution, represents 10–12% of the total DNA. Due to the relatively low concentration of this component at the end of the run, the accurate analysis of this small boundary is not possible; but the value of s may represent the average value of a DNA molecule mixture enriched in the smallest species relatively to the total DNA population present in the core particle. These results are in agreement with previous ones [14,15]. In our case, however, we never observed the 13 S material described by Stein [14] which would correspond to the binding of released histone octamers to non-dissociated core particles. This is probably due to the low concentrations of histones used in our study. At the maximal concentration of 210 μg/ml of core particles, the concentration of free histones cannot exceed 21

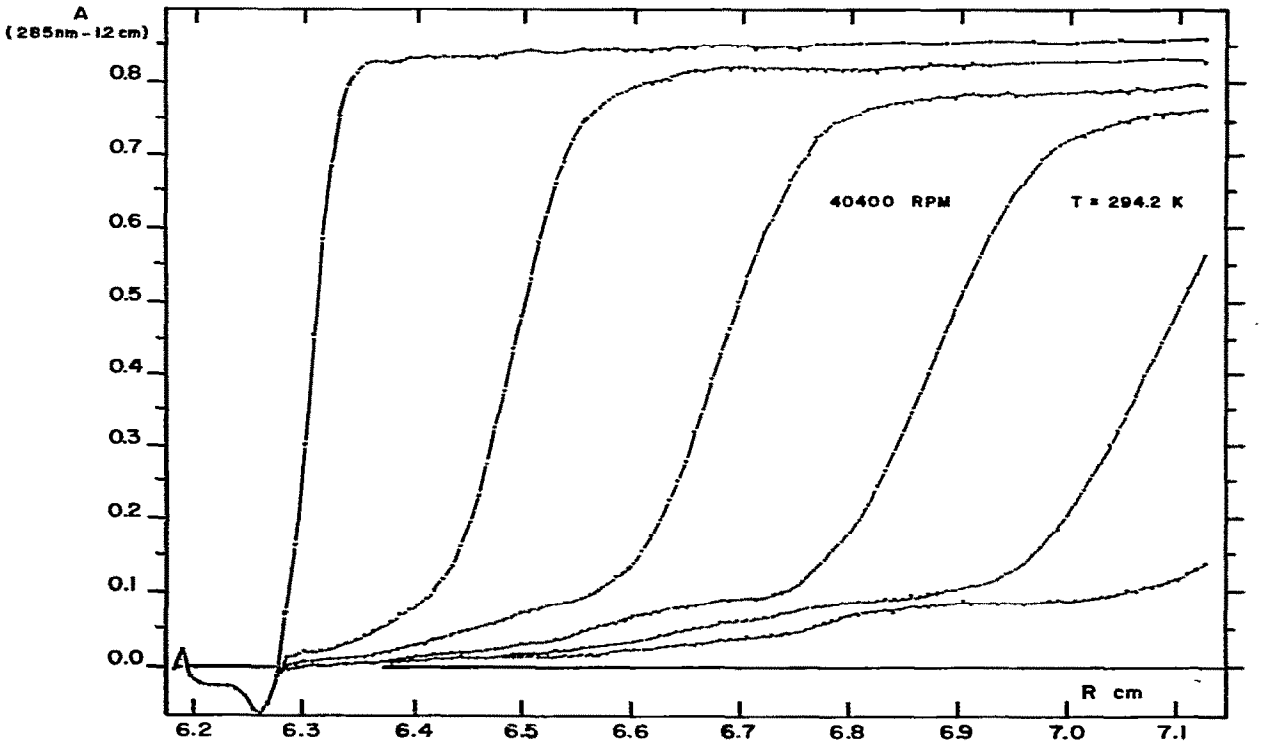

Fig. 3. Boundary sedimentation of core particle solution in buffer R + 0.6 M NaCl. The initial concentration was 175 μg/ml (O.D. 260 nm, 1 cm = 3.45). Each point of the concentration distribution is the mean value of 2^8 independent lectures of O.D. at each sampling position of the step motor scanning the image of the cell. Data are presented without any smoothing. $\Delta t = 1800$ s.

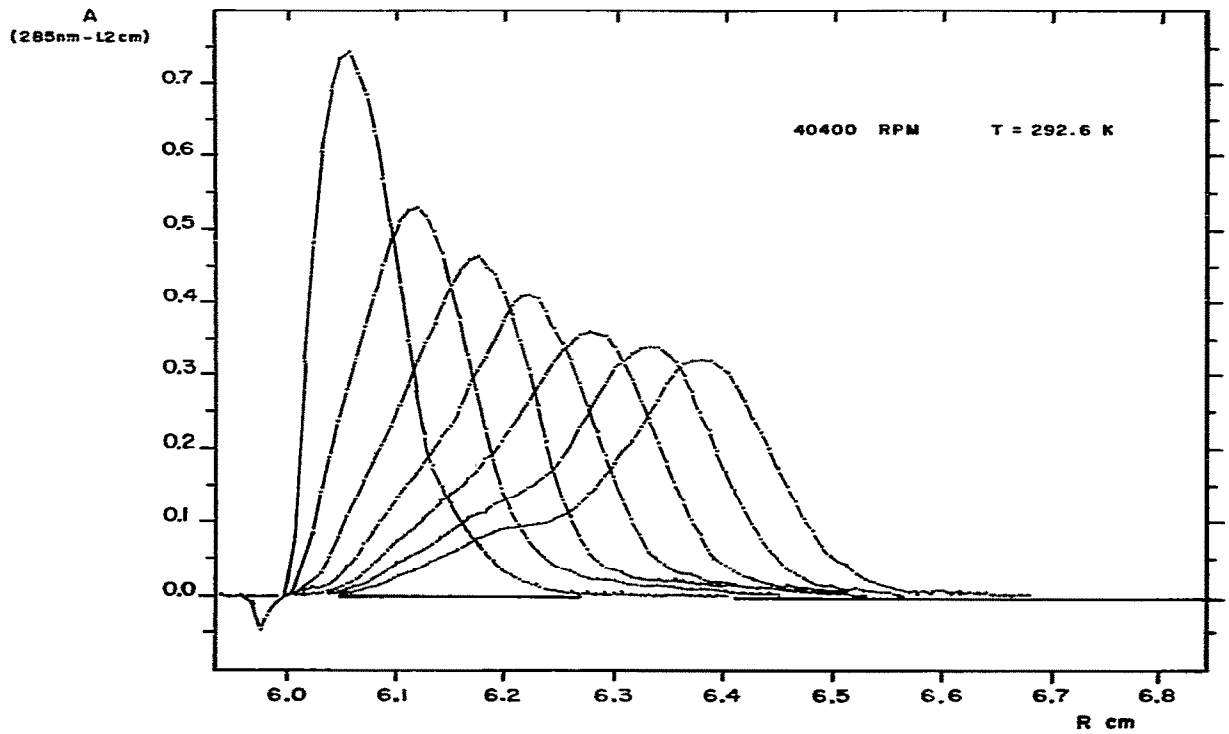

Fig. 4. Band sedimentation of core particle solution in buffer R + 0.6 M NaCl. The bulk solution is the same solvent in which 5% sucrose is added to ensure the stability of the band. The initial concentration of core particles was 175 μg/ml. $\Delta t = 600$ s. The conditions of data acquisition are the same as in fig. 3.

μg/ml. Under these conditions, the octamer, which was shown by Stein [14] to have a high affinity for core particles, cannot be present [16].

(b) *Band sedimentation* in 0.6 M NaCl also shows the presence of two components (fig. 4): a main band with $s_{20,w} = 9.26 \pm 0.18$ and a small band. In this case, the conversion of experimental results into standard ones leads to slightly less accurate values because one must take into account the error in determining relative viscosity and density of the bulk solution.

(c) By the *reduced second moment method* [17], it is possible to calculate the s/D ratio. This calculation, as shown in section 2.4, gives $D_{20,w} = 3.25 \times 10^{-7}$ cm$^2 \cdot$ s^{-1}. Assuming a partial specific volume of 0.659 [15], the molecular weight is $2.00 \pm 0.14 \times 10^5$, indicating that all the components of the core particle are still present stoicheiometrically in this band.

3.1.3. *Core particle partial unfolding is never observed in solution*

It is clear that, in 0.6 M NaCl, core particles behave in the same manner as in the presence of a high dye/phosphate ratio of ethidium bromide, with regard to microscopic and hydrodynamic criteria. These two destabilizing agents indeed have an effect in common, i.e., the presence, besides more or less unfolded core particles, of roughly 10% of naked DNA.

(a) We can ask ourselves immediately whether this situation reflects a true dissociation equilibrium of the core particle. To answer this question the following experiment was set up in the

case of ethidium bromide. After isolation of peak 2 (see fig. 2) and elimination of ethidium bromide by extensive dialysis, core particles were transferred to a 0.6 M NaCl medium and observed under the electron microscope. No naked DNA could be detected, but only a mixture of folded and partially unfolded core particles was present. The particles which completely dissociated when exposed to ethidium bromide have been definitely eliminated by sucrose gradient sedimentation, ruling out the possibility of dissociation equilibrium of the core particles.

(b) A contradiction, however, appears between the results obtained from circular dichroism or hydrodynamic measurement on the one hand and electron microscopy on the other.

In the case of circular dichroism, if 40% of the core particles had their DNA partially unfolded as shown by electron microscopy, it would have notably increased their total ellipticity value, giving an experimental value much higher than that observed.

A more detailed study of sedimentation data gives important information about the system.

In order to analyse the homogeneity of the observed fast band, we have simulated the band sedimentation of a mixture of 40% of 9.5 S and 60% of 10.8 S core particles, which corresponds to the relative amount of the two populations of core particles as observed in electron microscopy. Despite the apparent linear relationship between $\log c$ and $(r-r_m)^2$, the plot of the reduced second moment is not linear, indicating that the diffusion coefficient is not constant during the run. From the calculated mean value of D, the molecular weight was found to be only $\approx 60\%$ of the true value. This result obtained with two groups of core particles can be extended to any number of groups. In fact, the effect of polydispersity is to increase apparently the value of D, thus leading in any case

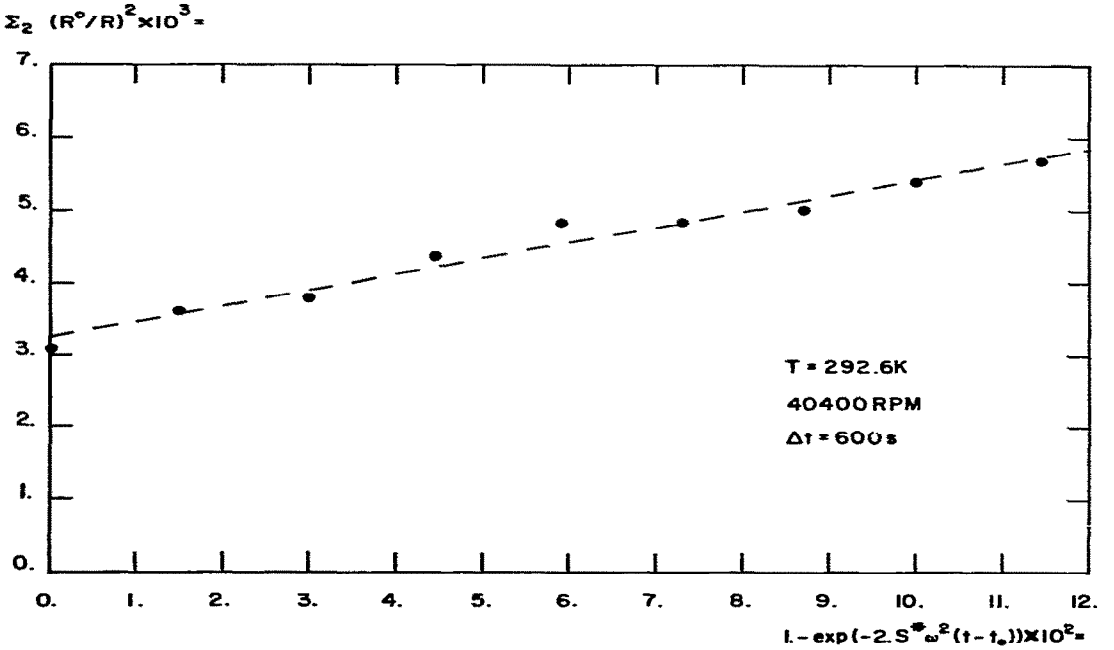

Fig. 5. Plot of the reduced second moment of mass versus $1-\exp[-2s^*\omega^2(t-t_0)]$. The slope of the linear relationship is $D^*/s^*\omega^2$. The linear least-square fit gives $a=2.1737\times10^{-2}$. $\omega^2=1.78783\times10^7\ s^{-2}$. The molecular weight is calculated by: $M=s^*RT/D^*(1-\bar{v}\rho)$.

to lower values for molecular weight. The corresponding analysis of our experimental data did not lead to such a conclusion and can be considered as a proof of the homogeneity of the core particle population under the fast band (fig. 5).

(c) The decrease in s from 10.8 to 9.5 S and of D from 3.9×10^{-7} $cm^2 \cdot s^{-1}$ [18] to 3.25×10^{-7} $cm^2 \cdot s^{-1}$ with a constant molecular weight can only result from a change in the shape of the particle.

In a first model we could assume that all of the DNA is unfolded with the eight histones remaining bound in a kind of quasi-linear complex. Such a model was proposed to explain the fluorescence properties in 0.6 M salt of core particles in which SH groups of H3 were labelled with fluorescent dye [20]. However, the s value of this model (calculated from the axial ratio, the molecular weight and the partial specific volume) is about 8 S in disagreement with the experimental value. Another line of evidence comes from circular dichroism. If DNA were unfolded with the histones acting only as counterions, the $|\theta|$ value would be much higher, since it is likely that a low value cannot be obtained in the absence of the specific DNA folding. As proposed by Cowman and Fasman [21], it might be the proximity of two coils of B-DNA which produces such a ψ-type low value. It was recently pointed out [15] that the change in the distance between SH groups of two H3 molecules might come from a structural modification of core particle induced by the fluorescent substituent.

In a second model, we have tried to explain the increase in the frictional coefficient by using a 'lollipop' model, suggested by electron microscope pictures. The radius, σ_1 of the head and σ_2, the radius of the tail of the lollipop were taken as 54 and 10 Å, respectively. Keeping $\sigma_1/\sigma_2 = 5.4$ constant and taking the length L of the tail as a variable, the f/f_0 ratio was calculated with an iterative process up to a value which differs only by 1×10^{-3} of the experimental value. The length L is found to be 130 Å, corresponding to an average of 40 base pairs [19].

Such a result is not consistent with circular dichroism data, since the increase in DNA molar ellipticity is fully explained by the presence of the 10% free DNA. It is no longer consistent with the DNAse I digestion pattern of the core particle in the salt range 0.1–0.7 M [15], indicating that the wrapping of the DNA is not significantly modified.

Therefore, the observed increase in the frictional coefficient of the core particle between low salt medium and 0.6 M NaCl cannot be explained by a partial unfolding of the DNA, but more likely, by a loosening of histone-DNA interactions. The N-terminal basic regions of core histones are known to be not necessary for maintaining the integrity of the core particle (22–24) in contrast with the central apolar parts, which are tightly bound to the DNA and play the essential role in the overall organization of the core particle. We can therefore assume, in 0.6 M NaCl, a sufficient screening of coulombic forces between DNA and N-terminal regions so that they can gain enough freedom to modify the hydrodynamic behaviour of the core particle. Such a conformation change was already postulated to explain NMR spectra [25].

It is thus clear that the partial DNA unfolding of some core particles is not present in solution but appears during the spreading on the positively charged grids. Electron microscopy reveals their latent unstability, which we must now explain.

3.2. Existence of two strong histone-DNA interaction zones at the ends of the core particle

3.2.1. How to interpret the differential destabilization of core particles

As mentioned above, non-histone proteins are removed with H1 and H5 by chromatography of the chromatin in 0.6 M NaCl. This ionic strength is much higher than necessary to dissociate the non-histone proteins. (Particularly, HMG 14 and HMG 17, which are known to interact with the core particle [26] are eluted by 0.35 M NaCl [27].) Such an explanation for the differential stability must then be rejected.

Histone acetylation also seems an unlikely explanation. We have recently studied (to be published) core particles reconstituted with DNA extracted from native core particles and the four histones among which H3 and H4 were either

strictly non-acetylated or monoacetylated *. Both sets have the same behaviour in 0.6 M NaCl as native core particles, ruling out any potential role of histone acetylation in the observed differential behaviour of core particles.

It is possible to interpret previous results if there are two strong histone-DNA interaction zones of only two or three base pairs at the ends of the 146-base-pair core particle. The preservation of these short regions depends on the micrococcal nuclease attack during core particle preparation. It is relatively infrequent that both of these strong interaction zones are cleaved in the same core particle; the frequency of such an event which leads to complete unfolding of the DNA in destabilizing processes is only 10%. In 40% of the cases, only one zone is attacked, which leads to a polar destabilization of the core particles and to a partial unfolding of their DNA during spreading on the electron microscopy grids. The remaining 50% of the core particles have both of their extremities protected and resist unfolding when exposed to destabilizing agents. Such an interpretation is in agreement with the recent study of McGhee and Felsenfeld [3] who have shown that only a few of the terminal phosphates of the core particle DNA are involved in intimate charge-charge interactions with histones.

3.2.2. Electrophoretic analysis of the DNA lengths

In line with this interpretation, electrophoretic analysis of the lengths of the DNA was performed. DNA from peak 1 (coming from the dissociation of 10% core particles in consequence of ethidium bromide intercalation) was compared with DNA extracted from core particles constituting peak 2 (those which are resistant either completely or partially to the ethidium bromide-induced destabilization). Four channels in a 10% polyacrylamide long gel were employed. The two species of DNA were deposited in two channels and each of these two species, intermixed with a set of markers (Hinf 1 restriction fragments from Ca MV DNA), was deposited in an adjacent channel. After scanning of the gel, the mean length of each kind of DNA was calculated by comparison with the markers in each channel (fig. 6). The values obtained are 142 base pairs for peak-1 DNA and 145 base pairs for peak-2 DNA. In order to estimate the polydispersity of each DNA, a correction was applied to take into account the diffusion process as follows:

(a) The variance of the band due to the diffusion has been estimated for a strictly monodisperse DNA marker.

(b) The mass and shape of the marker and core particle DNA are close enough to assume the diffusion process gives an identical variance in both cases.

(c) The distribution of species in each band is a normal distribution, and it is possible to assume that the total variance V_T of the band is the sum of the variance V_D due to the diffusion and the variance V_P due to the polydispersity.

As a result, the marker 'H_{11}' (233 base pairs) has a variance of 21 mm^2 on the scan. The variance V_T of peak-1 DNA band is 25 mm^2. One can then deduce $V_P = V_T - V_D = 25 - 21 = 4$ mm^2 and σ_P, the standard deviation. The half width of the band in base pairs is given by $\Delta n = m(e^{\sigma_P a} - 1)$ where m is the mean length, σ_P, the standard deviation calculated from the variance V_P, and a, the slope of the linear relationship (see fig. 6). Thus, the distribution for peak-1 DNA is equal to 142 ± 1 base pairs. The variance V_T of peak-2 DNA band is 46 mm^2. As mentioned above, one can calculate V_P, σ_P and Δn. The distribution for peak-2 DNA is equal to 145 ± 3 base pairs.

These results mean that the difference between the length of a DNA piece which can ensure the two 'locking' zones and that of a DNA which cannot furnish any of them is very small but significant. If one assumes the necessary length of the core particle DNA to be 146 base pairs [28,29], this difference is only four base pairs; in other words, two base pairs, at each end of the core particle DNA, strongly interact with histones. This stabilizes core particle when exposed to some perturbating agents.

* Kindly provided by P. Sautière (I.R.C., Lille).

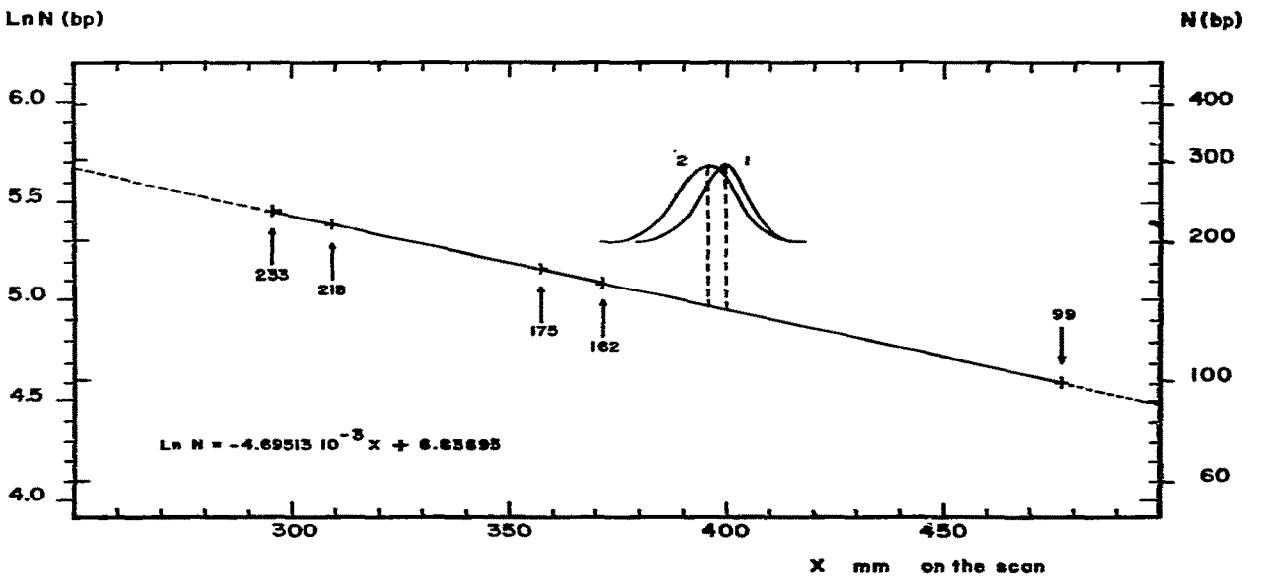

Fig. 6. Electrophoretic analysis of the lengths of peak 1 and 2 DNA. These lengths are determined from the line established with the migration of the markers (Hinf I restriction fragments of Ca MV). To compare the two DNA bands, each has been redrawn, to the same scale, on a marker set taken as reference.

4. Conclusion

We assume negatively charged phosphate groups situated at positions 1 and 2 in one end and at positions 145 and 146 in the other end to play an essential role in the stability of the core particle. When two sites are eliminated from one end, it creates a latent instability at this extremity. When the four sites are eliminated (an event occurring for about 10% of the core particles), the DNA is completely removed from the histone core and is found free in solution. Of course, a series of intermediate digests can occur, which explains the width of both peak 1 (free DNA) and peak 2 (core DNA) as measured by electrophoresis (see fig. 6). Some DNA species of peak 1 can migrate at the same level as some DNA species of peak 2. If, according to recent structural studies [30], a positively charged helical ramp at the surface of the core organizes the wrapping of DNA in less than two superhelical turns, it appears that the end points of the 146-base-pair DNA act as anchoring regions.

Acknowledgements

The technical assistance of J. Dunand and D. Buhr was highly appreciated. We thank H. Guilley (Virology of I.B.M.C., Strasbourg) for his gift of Hinf I restriction fragments of Cauliflower Mosaic Virus and G. Daney de Marcillac for his help in discussing the applied mathematical methods for data analysis. We thank S. Schmid for his careful reading of the manuscript, regarding correction of the english.

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