

Polyamine addition to preparation media induces chromatin condensation, irreversibly at low ionic strength

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Polyamines (spermine, spermidine) are commonly used as stabilizing cations in the chromatin preparation media. Their residual binding to chromatin is not easily reversed at low ionic strength, even after extensive dialysis, as evidenced by the use of labelled spermidine. Electric dichroism measurements show that their presence interferes with the physico-chemical characterization of chromatin by maintaining a condensed structure. These results give a definite answer to the controversy about the sign of optical anisotropy of chromatin determined by electric and flow dichroism techniques.

Chromatin; Polyamine; Electric dichroism

1. INTRODUCTION

In 1973, Hewish and Burgoyne [1] evidenced the nucleosomal structure of rat liver chromatin that they had isolated using polyamines as stabilizing cations. Since that time, several protocols for the preparation of chromatin and core particles from different organisms included spermine and spermidine [2–7]. It was recently shown that multivalent cations could produce chromatin condensation and stabilization of superstructures. We followed this condensation process using electric linear dichroism (ELD), a technique particularly sensitive to conformational changes [8,9]. We observed a reversal of dichroism sign (from negative to positive) when polyamines induced the condensation of chicken erythrocyte (CE) chromatin.

Unfortunately, much controversy still remains concerning the sign, amplitude of optical anisotropy of whole chromatin and its variation with ionic strength. This open question has been debated to variable extent in many recent papers and review articles without receiving any definitive answer [8,11–16]. At low ionic strength (≤ 1 mM NaCl), the optical anisotropy was most frequently found to be negative under flow [13,17,18] and electric field orientation forces [8,11,19–23]. In opposition with these findings,

Marion et al. [3–5] consistently reported positive electric birefringence for oligonucleosomal chains of more than 6 nucleosomes in length and for whole chromatin when histone H1 was present. In Crothers' group [24–26], positive ELD was evidenced on covalently modified (cross-linked) chromatin. Using FLD, Dimitrov et al. [13] and Makarov et al. [18] found positive dichroism at a salt concentration higher than 2 mM NaCl. Likewise, Tjerneld et al. [6] also observed positive FLD on unsheared mild digested chromatin prepared in the presence of polyamines.

In this paper, we clearly demonstrate that the presence of polyamines in all the buffers used throughout the preparation of chromatin maintains a condensed structure similar to that obtained when an adequate amount of spermine or magnesium is added to native chromatin and induces its first condensation step. We also show that the difficulty to remove spermine and spermidine bound to chromatin is responsible for this effect.

2. MATERIALS AND METHODS

Chicken erythrocyte chromatin (CE chromatin) of 10–80 nucleosomes long was prepared from isolated nuclei by mild micrococcal nuclease digestion using standard procedures previously described [27], except that, after digestion, the pellet was resuspended twice in a cleaning buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.5 mM PMSF, pH 7.5) and centrifuged at $300 \times g$ for 5 min. The final pellet was resuspended in the lysis buffer (5 mM Tris, 1 mM EDTA, 0.5 mM PMSF, pH 7.5) and dialyzed overnight against this buffer to complete the lysis. The last centrifugation step was made at $20000 \times g$ for 15 min and the supernatant, extensively dialyzed against 1 mM cacodylate buffer, pH 6.5, was the starting material for our measurements. In some preparations, 0.5 mM spermidine +

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Abbreviations: CE, chicken erythrocyte; ELD, electric linear dichroism; PMSF, phenylmethylsulfonylfluoride; FLD, flow linear dichroism; Sp/P, spermine over mononucleotide molar ratio

0.15 mM spermine were added to the nuclei suspension medium and maintained present through all preparation steps, except at the final dialysis stage. To follow the fate of spermidine, [^3H]spermidine at 100 $\mu\text{Ci}/1$ (New England Nuclear, 1 mCi/ml) was incorporated in the spermidine + spermine mixture added.

The chromatin precipitation curves were determined as previously described [8].

All electric dichroism (ELD) measurements were performed in an air-conditioned room at 20°C under a 10 mm pathlength as previously described [11].

Dissociation-reassociation experiments of chromatin were made in the following way: native chromatin sample was dialyzed against 0.5 M NaCl in 1 mM cacodylate buffer, pH 6.5, then against the same buffer without NaCl, the sample remaining in the dialysis bag.

3. RESULTS

From our recent results on CE chromatin condensation by spermine, spermidine and MgCl_2 [8], we suspected that positive birefringence values obtained by Marion et al. [3–5] on rat liver chromatin could be related to the presence of polyamines in their preparation protocols. If we incorporated such polyamines in the preparation media, the ELD results, even after extensive dialysis against 1 mM cacodylate, pH 6.5, were particularly revealing: the dichroism of native chromatin displayed a positive value at low field strengths (up to about 6 kV/cm) and became negative at higher fields (fig.1b). The titration of this chromatin by spermine or Mg^{2+} induced a positive dichroism sign in the whole field strength range at lower Sp/P ratios or Mg^{2+} concentrations than when no polyamines were present in the preparation buffers (fig.1).

Such a comparison raised a new question: may spermine and/or spermidine still be bound to chromatin after extensive dialysis and be responsible for a partial condensation? We answered this question by incorporating [^3H]spermidine in our preparation protocol. After three dialyses of the digested nuclei against their lysis buffer, no more radioactivity was present in the external dialyzing bath. After the last centrifugation (see section 2), native chromatin had a radioactivity of 75300 dpm/ml or 125 dpm/ μg DNA as chromatin had a final DNA concentration of 600 $\mu\text{g}/\text{ml}$. During the three subsequent dialyses against 1 mM cacodylate buffer, pH 6.5 (one of them overnight), no more radioactivity was removed. Taking into account the ratio of unlabelled over labelled spermidine, one could estimate that, in such a case, about 30% of spermidine was still bound to chromatin. This ^3H -labelled polyamine-containing chromatin displayed precipitation profiles (absorbance at 260 nm, A_{260} , versus Sp/P ratio or MgCl_2 concentration) similar to those of native chromatin with the 50% precipitation point displaced to lower Sp/P or MgCl_2 concentrations (data not shown). The dependence of reduced electric dichroism $\Delta A/A$ of this chromatin on MgCl_2 concentration at 6 kV/cm had a trend similar to that previously described for native chromatin without polyamine addition [9]. However, as seen from the comparison of fig.2a

and b, the reversal of sign occurred at a lower MgCl_2 concentration (25 μM) with respect to about 500 μM without polyamine. The intermediate levelling-off of dichroism was present in the positive range (fig.2b) rather than in the negative one (fig.2a). These results provide evidence for a more condensed structure of the polyamines-containing chromatin. X-ray scattering measurements of Koch et al. (submitted for publication) confirmed our results. The dependence of relaxation times of this chromatin on MgCl_2 concentration is similar to that of native chromatin previously described [9,10].

Is it possible to definitively remove polyamines to avoid their interference in the physico-chemical characterization of chromatin samples? To answer this question, we performed precipitation experiments on two radiolabelled chromatin samples as a function of NaCl concentration (0–600 mM). Radioactivity was measured in the supernatants. It is well known that the increase of ionic strength (up to about 0.5 M NaCl) of chromatin solutions induces the dissociation of H1 or H1/H5 histone(s) from chromatin. The first sample (prepared as described in section 2) showed a classical

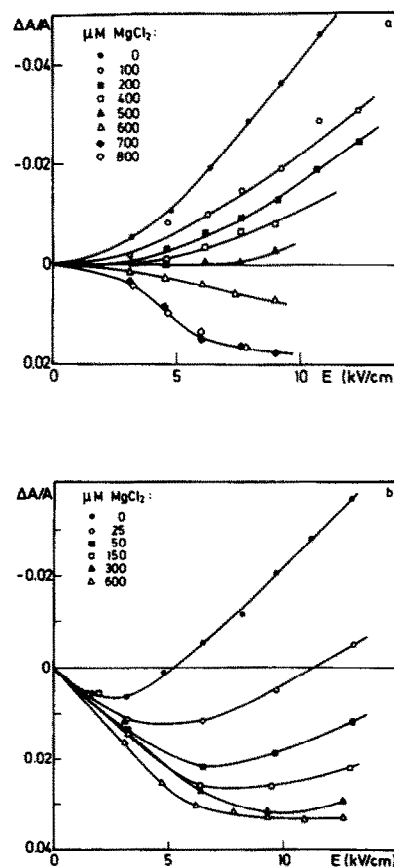


Fig.1. Field strength dependence of the reduced electric dichroism of CE chromatin at 260 nm for increasing MgCl_2 concentrations in 1 mM cacodylate buffer, pH 6.5, without (a) and with (b) spermine + spermidine in the preparation media.

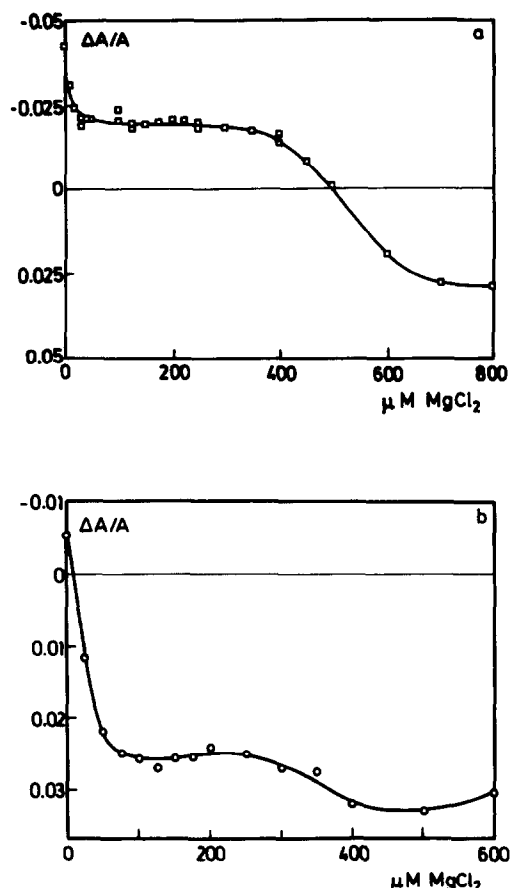


Fig.2. Reduced electric dichroism (at 6 kV/cm and 260 nm) of CE chromatin as a function of MgCl_2 concentration without (a) and with (b) spermine + spermidine in the preparation media.

behaviour (minimal solubility around 240 mM) and the supernatant radioactivity remained constant throughout the whole ionic strength range. Indeed, our measurements were made at 120, 240, 360, 480 and 600 mM NaCl and at these concentrations, polyamines were dissociated from chromatin and were not present in the precipitate. Let us recall that Stone et al. [7] succeeded in removing polyamines from *Physarum polycephalum* core particles using CM-Sephadex C-25 in the presence of 50 mM NaCl. The second sample was dialyzed first against 0.5 M NaCl and then against a 1 mM cacodylate buffer, pH 6.5 (without being removed from the dialysis bag). In this case, no more radioactivity was retained in the supernatants. This demonstrated that spermine and spermidine were removed from chromatin during these two dialysis steps. The ELD measurements (fig.3) confirmed these ideas: the first sample displayed the classical dependence of $\Delta A/A$ versus electric field E for a polyamine-containing chromatin, i.e. positive dichroism at low field strengths and reversal to negative values at higher fields. On the contrary, the ELD of the doubly dialyzed sample is always negative and similar to that obtained without using polyamines in the preparation buffers.

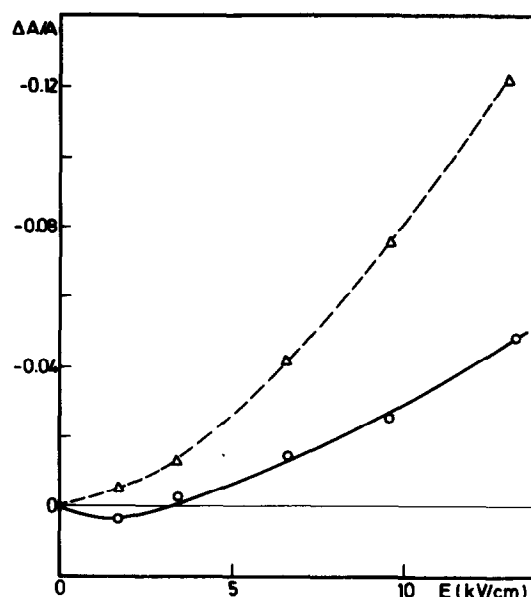


Fig.3. Field strength dependence of the reduced electric dichroism of CE chromatin at 260 nm. (\circ) Chromatin with spermine + spermidine in the preparation media; (Δ) same sample as (\circ) dialyzed first against 1 mM cacodylate buffer, pH 6.5, with 0.5 M NaCl, then against the same buffer without NaCl added.

4. DISCUSSION AND CONCLUSIONS

The above experiments yield important conclusions in two regards. First, the use of [^3H]spermidine gave an unequivocal support to the presumptions of Makarov et al. [18] that addition of spermine and spermidine to chromatin preparation media changed the negative FLD signal to a positive one. We also evidenced that polyamine binding to chromatin is not easily reversed at low ionic strength (see also [28]). For this reason, concentrations of polyamines must be carefully controlled in experiments on chromatin structure if interference is to be avoided. This naturally leads us to the second point: our experiments throw more light on the problem of the conflicting results about the sign of optical anisotropy. While, in our hands, all the native chromatins we studied displayed negative dichroism, Marion et al. [3] found a positive sign for the optical anisotropy of rat liver chromatin longer than 6 nucleosomes. If we recall that Marion et al. used spermine + spermidine in their preparation media and took measurements in electric fields < 4 kV/cm, their results are compatible with ours when using similar experimental conditions.

On the same basis, the divergent birefringence results of Marion's group [5,29] on reconstituted chromatin samples can be explained. Indeed, Roche et al. [29] and Marion et al. [5] measured positive and negative birefringence using 10 mM Tris and 1 mM phosphate buffers, respectively. We have good experimental reasons to believe that this discrepancy arose from the ionic

strength used for the measurements, as Marquet et al. [30], by ELD on one hand, and Dimitrov et al. [13] by FLD, on the other, evidenced a sign change (from negative to positive) around 4 mM NaCl. In opposition to Dimitrov et al. [13], we do not think that negative optical anisotropy of native chromatin at low fields can be explained by a lysis of the nuclei at very low ionic strength (≤ 2 mM NaCl) since Marion et al. [3] also used a low ionic strength buffer, namely 0.2 mM EDTA, for the nuclei lysis.

As shown above, H1/H5 removal from chromatin by increasing the ionic strength up to 0.5 M NaCl produced the dissociation of spermine and spermidine from chromatin in agreement with recent experiments of Stone et al. [7].

In conclusion, although polyamines are commonly used as stabilizing cations in several preparation or crystallization media, the greatest caution has to be taken before any interpretation of physico-chemical measurements on chromatin can be made if polyamines are present [7,28]. In this context, the use of labelled spermidine in our experiments gives a definite answer to the controversy about the sign of the optical anisotropy of chromatin samples.

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