

Chromatin superstructure

A study with an immobilized trypsin

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Hen erythrocyte chromatin was treated with trypsin immobilized on collagen membranes and the unfolding of chromatin fiber was followed by light scattering at 90° and flow linear dichroism. Chromatin was found almost completely decondensed when the bulk of H1 and H5 was digested while H3 was still intact. Further digestion leading to degradation of both H3 and the rest of H1 and H5 accounted for no more than 10–15% of the total effect. When chromatin with trypsin-cleaved H1 and H5 was titrated with increasing amounts of spermidine it folded similarly to the control sample. This finding suggests that charge neutralization appears a likely mechanism for maintaining the structure of the 30 nm chromatin fiber by the C-terminal domain of H1 and H5.

Chromatin structure Immobilized enzyme Trypsin Light scattering Flow linear dichroism

1. INTRODUCTION

Histone H1 is known to play a fundamental role in chromatin both at the nucleosome level and in maintaining its higher order structure [1–3]. Most of the evidence has come from electron microscopy and physical studies of chromatin after removal and subsequent reassociation of histone H1. In many cases the elimination of H1 has been achieved by digestion of chromatin with trypsin in solution [4–6]. A serious disadvantage of this approach is that the degradation of H1 and H3 proceeds with a similar rate and one cannot discriminate the contribution of each one of these histones in stabilizing the structure of the chromatin fiber. This drawback was overcome by using trypsin immobilized on a collagen membrane [7,8]. When chromatin was digested under such conditions and the unfolding of chromatin was

followed by electric birefringence and circular dichroism, Marion et al. [7,8] questioned the accepted view that H1 is essential in maintaining the chromatin superstructure. They claimed that cleavage of H1 did not affect chromatin structure and that the unfolding occurred only after digestion of the terminal regions of H3. An analogous conclusion was made by Saccone et al. [9] using trypsin conjugated to soluble polyacrylamide: the condensation of chromatin correlated with H3 degradation better than H1 and H5. Here we present our results obtained by following the changes in light scattering at 90° and in flow LD upon digestion of hen erythrocyte chromatin with an immobilized trypsin. Chromatin was found almost completely unfolded when the bulk of H1 and H5 was degraded while H3 was still intact.

2. MATERIALS AND METHODS

Hen erythrocyte nuclei were prepared as described in [10] and used to isolate chromatin by mild treatment with micrococcal nuclease and

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Abbreviation: LD, linear dichroism

overnight dialysis against 3 mM Tris-HCl, pH 7, 2 mM NaCl and 0.25 mM EDTA. After centrifugation at $10000 \times g$ for 5 min the supernatant was used in all experiments.

Proteolytic degradation was carried out using trypsin (Sigma, TPKC-treated) covalently bound to the surface of water insoluble collagen membrane (Centre technique du Cuir, Lyon, France) as described in [8]. Digestion was initiated by dipping the membranes (0.5/cm) into chromatin solution (6–8 ml, 8–10 A_{260} per ml). Agitation and temperature control (24°C) were ensured using a giratory rotor bath shaker. A control sample with no membranes added was processed in parallel to check that no detectable endogenous proteolysis occurred. Aliquots were taken at selected time intervals and used for histone electrophoresis, LD and light scattering measurements.

In the reconstitution experiments with spermidine, trypsinized chromatin was extensively dialysed against 100 mM NaCl, 10 mM Tris-HCl, pH 7, and 0.25 mM EDTA to remove the histone fragments cleaved by the enzyme [3]. Chromatin was then dialysed against 3 mM Tris-HCl and

0.25 mM EDTA and titrated with increasing amounts of spermidine under gentle agitation.

LD measurements were carried out with a Jobin and Yvon mark III dichrograph equipped with an achromatic quarter wavelength device, allowing a direct reading of LD spectra. A previous study details the method [11].

Light scattering experiments were performed with Aminco SPF1000 spectrofluorimeter. The variations of the scattered light were measured at 90° to the monochromatic beam ($\lambda = 350$ nm) using a fluorescence cuvette.

Electrophoresis of histones was carried out according to Laemmli [12].

3. RESULTS AND DISCUSSION

Fig.1 clearly shows that H1 and H5 are the first histones to be cleaved upon digestion of chromatin with an immobilized trypsin, followed by H3. The possibility of degrading H1 and H5 while keeping H3 intact offers a good approach to discriminate the contribution of H1 + H5 and H3 in maintaining the structure of the 30 nm chromatin fiber.

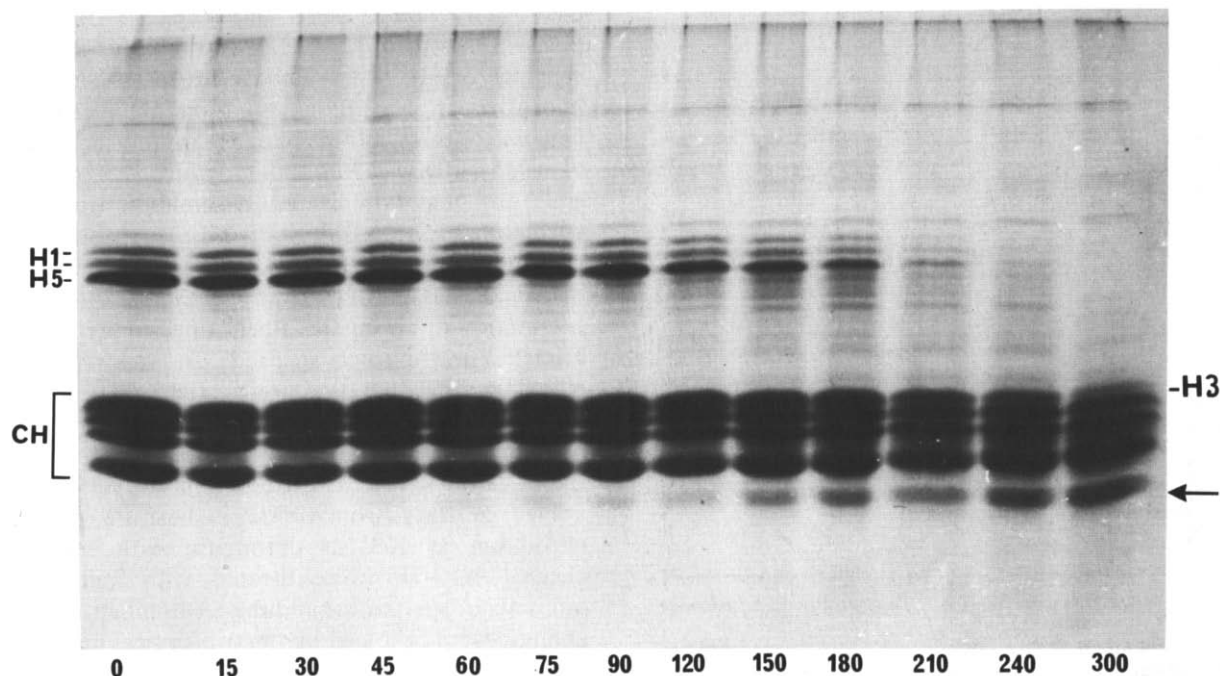


Fig.1. 18% polyacrylamide-SDS electrophoresis of histones extracted from hen erythrocyte chromatin, digested with an immobilized trypsin for the times indicated (in min). The arrow shows the trypsin-resistant peptide of H5 [18]. CH, core histones.

Two parameters of the latter, the extent of its condensation as well as the orientation of the faces of nucleosome discs relative to the fiber axis, were investigated by following the changes in light scattering at 90° and the flow LD upon digestion of chromatin from hen erythrocyte with an immobilized trypsin. As it was recently shown [13,14], the intensity of the scattered light at 90° ($I/90^\circ$) reflected the extent of condensation of the chromatin fiber. Fig.2 summarizes the changes in $I/90^\circ$ and in the reduced LD upon trypsinization of chromatin in 80 mM NaCl, 10 mM Tris-HCl, pH 7, and 0.25 mM EDTA. The intensity of the scattered light progressively decreased while the reduced LD ($\Delta A/A$) at 258 nm increased, both reaching a plateau after approximately equal time of digestion (about 3 h). When these changes were compared to the respective digestion pattern of histones (see fig.1) it became clear that almost the

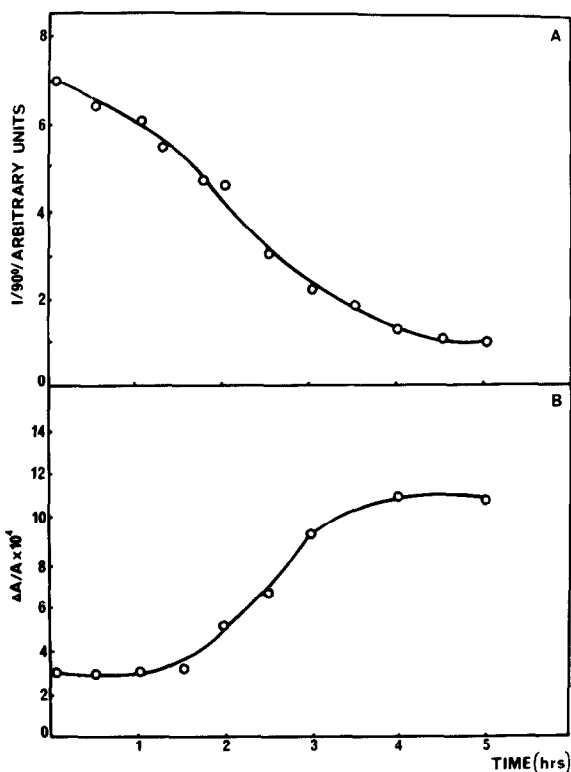


Fig.2. Changes in the intensity of the scattered light at 90° ($I/90^\circ$) (A) and in the reduced LD at 258 nm (B) upon digestion of hen erythrocyte chromatin in 80 mM NaCl, 10 mM Tris-HCl, pH 7, and 0.25 mM EDTA with immobilized trypsin for the times indicated.

whole amplitude of the changes in $I/90^\circ$ and in LD resulted from hydrolysis of H1 + H5 only: H3 and the other core histones were still intact. Further digestion, when H3 was degraded, accounted for not more than 10–15% of the total effect. These figures, however, cannot be attributed to H3 only, since its initial digestion coincided with the degradation of the rest of H1 and H5. The data obtained were solely due to trypsinization since no sign of endonuclease activity was mentioned even after a prolonged incubation with the membranes (not shown). These findings demonstrate that the unfolding of chromatin is due mainly to the cleavage of H1 and H5, the role of H3, if any, being negligible in this respect.

The positive LD at 258 nm increased more than twice upon trypsinization due to the increase in the linear dimensions of the fibers as a result of their unfolding [11]. As we showed recently, the role of H1 in maintaining the positive anisotropy of chromatin was accomplished by its globular part alone [6]. These findings suggest the same role for the globular domain of H5.

The experimental data reported here contradict those obtained by Marion et al. [7,8]. Using electric birefringence, these authors have found a positive anisotropy of chromatin after tryptic cleavage of H1, which changed to negative upon hydrolysis of H3. Based on these findings they concluded that contrary to the widely accepted view [15,16] it was H3, not H1, which was responsible for maintaining the chromatin structure: chromatin unfolded only after digestion of the terminal regions of H3. Our explanation of this discrepancy is the use of an electric field for the orientation of chromatin fibers when applying the electric birefringence method. It was reported elsewhere [17] that the electric field may induce structural alterations in chromatin.

With the aim to gain insight into the mechanism by which the non-structured domains of H1 are involved in the chromatin superstructure, native chromatin as well as chromatin with trypsin-cleaved H1 + H5 were titrated with increasing amounts of spermidine and the resultant structural changes were followed by the two optical methods (fig.3). In the case of native chromatin, the increase of the polyamine concentration caused an increase in $I/90^\circ$ with a maximum at about 3.5×10^{-5} M, where the intensity of the scattered light

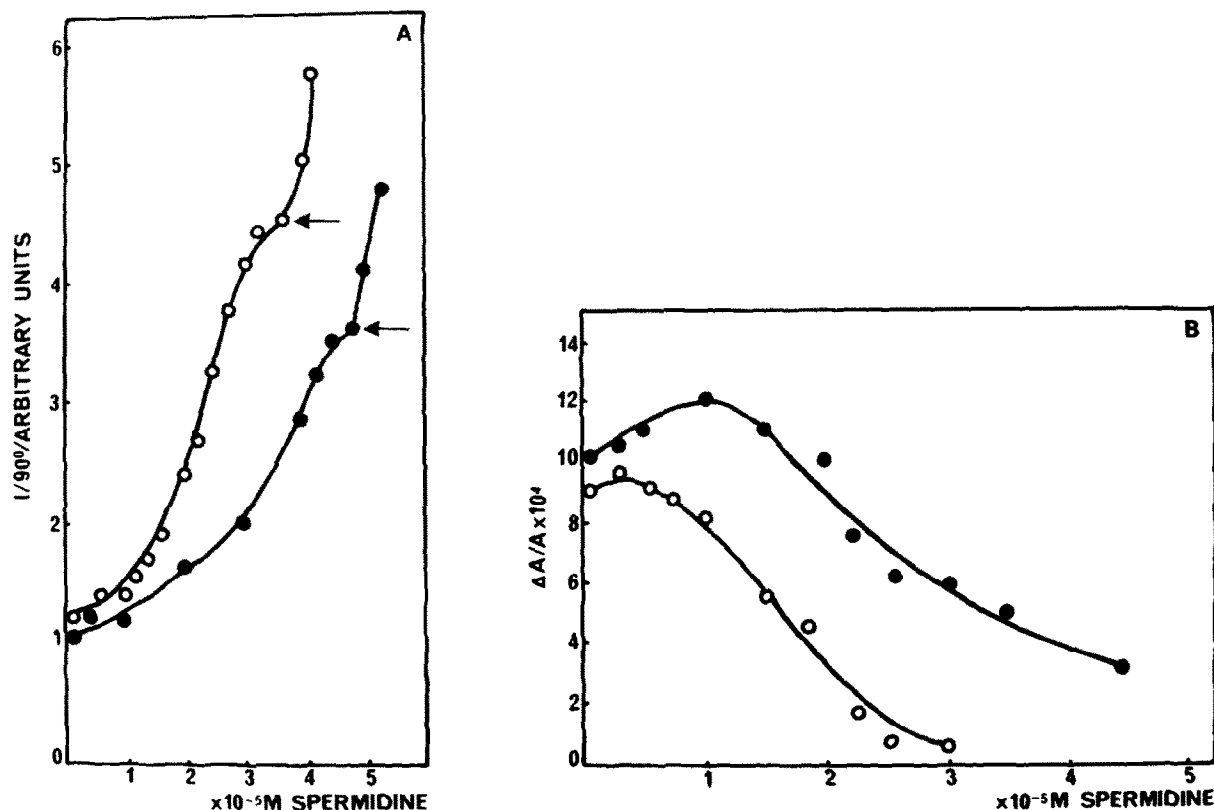


Fig.3. Changes in the intensity of the scattered light at 90° ($I/90^\circ$) (A) and in the reduced LD at 258 nm (B) upon titration with spermidine of control hen erythrocyte chromatin ($\circ-\circ$) and chromatin digested in 80 mM NaCl, 10 mM Tris-HCl, pH 7, and 0.25 mM EDTA with immobilized trypsin for 3 h ($\bullet-\bullet$). Before titration chromatin samples were extensively dialysed against 3 mM Tris-HCl and 0.25 mM EDTA. The arrow indicates the last point before the aggregation began.

was 4-times higher than the initial value. Further increase of spermidine concentration resulted in a sharp increase in $I/90^\circ$. A similar dependence of $I/90^\circ$ was reported upon titration of chromatin with divalent cations [13]. The gradual increase of $I/90^\circ$ observed up to 3.5×10^{-5} M spermidine is an intramolecular process, which reflects the condensation of chromatin, while the following sharp increase in $I/90^\circ$ is an intermolecular event, connected with the aggregation of the fibers [13]. Trypsin-cleaved chromatin behaved slightly differently: the maximum increase in $I/90^\circ$ before the aggregation took place did not exceed 3–3.5-times the respective initial value and was observed at a higher spermidine concentration (4.5×10^{-5} M). As for LD measurements both native and trypsinized chromatin behaved similarly upon increasing spermidine concentrations. All these data sug-

gest that chromatin with cleaved terminal parts of H1 and H5 have undergone polyamine-induced folding in a way similar to that of the native chromatin: the orientation of nucleosomes is preserved but the final structure of the fiber of the cleaved chromatin is less compact and is reached at higher polyamine concentrations.

The C-terminal domain of H1 appears to be the most likely region responsible for the condensation of the nucleofilament [3]. Our data on titration of trypsin-digested chromatin with spermidine show that this condensation is accomplished in a way similar to that of the control chromatin, i.e. the condensation in the presence of polyamine does not need an intact C-terminal region. It follows, therefore, that the polyamines mimic the role of this region of H1 in chromatin condensation, most probably by neutralizing the negative charges of

the phosphates. Assuming non-specific interactions between DNA and polyamines, charge neutralization appears a likely mechanism for maintaining the structure of the 30 nm chromatin fiber by the C-terminal domain of H1.

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