Binding of Polylysine and Ethidium Bromide to Nucleosomal DNA: Comparison of Biochemical and Electron Microscopical Results

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Ethidium bromide and polylysine interact with nucleosomal DNA and lead to changes of biochemical properties and to morphological changes as to the distance between the two core particles of a nucleosome dimer.

With increasing polylysine concentration, the buoyant density of nucleosomes decreases and the accessibility of the nucleosomal DNA to micrococcal nuclease is lowered. Electron microscopy of polylysine treated nucleosome dimers reveals a shortening of the internucleosomal distance as compared with controls.

Treatment of nucleosomes with ethidium bromide leads to an enhanced accessibility of the nucleosomal DNA to micrococcal nuclease. Electron microscopy reveals an increase in length of the DNA connecting the two nucleosome cores in the presence of the dye.

Both the binding of polylysine and the treatment with ethidium bromide apparently do not affect the histone arrangement within the nucleosome core as suggested by chemical cross-linking of histones and DNA with formaldehyde, and no obvious morphological changes of the nucleosome cores can be observed.

Interphase chromosomes in eukaryotes are organized as an ordered array of subunit particles (for review, see [1]). These histone/DNA complexes, termed nucleosomes [2], consist of a core histone octamer with 146 nucleotide pairs of DNA and a linker DNA region of variable length, depending on the tissue from which the chromatin is prepared [3].

The tools which led to the elucidation of this structure have been electron microscopy [2, 4–6], X-ray diffraction [7, 8] and enzymatic cleavage with micrococcal nuclease [9], DNase I [10], DNase II [11] and, initially, an endogenous Ca/Mg-dependent nuclear endonuclease [12]. In addition, chemical cross-linking procedures [7, 13–17] have been used to describe the sites of contact between histones and histones [13–16] and histones and DNA [17].

Additional methods are based on interactions of exogenous molecules with the nucleosomal DNA such as the polycation polylysine, which binds to available phosphate groups of the DNA [18, 19] or such as ethidium bromide which intercalates into the nucleosomal core DNA (*e. g.* [20–25]) and binds electrostatically at the internucleosomal linker DNA region [25].

The current knowledge about the nucleosome structure is a good example for a mutual complementation of biochemical and electron microscopical data. In this paper we extend this approach and combine a further analysis of both the polylysine and ethidium bromide binding phenomena with electron microscopy. The results from chemical cross-linking studies and enzymatic digestions are compared with the morphological appearance of the modified nucleosome particles in the electron microscope.

The biochemical studies were done with nucleosome monomers, dimers and trimers (with equal results) whereas the morphological investigations were done with nucleosome dimers as the smallest suitable model for the chromosomal chain of nucleosomes.

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Materials and Methods

Materials

Micrococcal nuclease (EC 3.1.4.7) was from Boehringer (Mannheim, FRG). Nuclease free sucrose, ethidium bromide, glutaraldehyde and all reagents for electrophoresis were from Serva (Heidelberg, FRG). Polylysine hydrobromide ($M_r = 3400$) was from Sigma (München, FRG). BAC (Benzyldimethylalkylammonium chloride) was kindly provided by Bayer AG (Leverkusen, FRG). All other reagents were (reagent grade) from Merck AG (Darmstadt, FRG).

Preparation of nucleosomes and nucleosome oligomers

Nucleosome monomers, dimers and trimers were prepared from rat liver nuclei in buffer A [12] as previously described [26] by repeated centrifugation, dialyzed against buffer B (5 mM sodium phosphate buffer, pH 6.8, 0.2 mM Na_2EDTA) and subsequently analyzed under the conditions of the respective experiments.

Formaldehyde fixation

Isolated nucleosomes or their oligomers in buffer B were dialyzed for 12 h against buffer B containing 1% formaldehyde (w/v) followed by exhaustive dialysis against formaldehyde free buffer B for 48 h [27, 28].

Formaldehyde cross-linking was either followed by CsCl gradient centrifugation or (in the case of ethidium bromide treatment in combination with nuclease digestion) cross-linking was followed by exhaustive dialysis against double distilled water, lyophilization and electrophoresis (see below).

Cesium chloride gradients

Formaldehyde fixed nucleosomes in buffer B and crystalline cesium chloride were mixed at the appropriate ratio needed in the respective experiment, paraffin was layered on top and the mixture was run in the Beckman SW 60 rotor at 33000 rpm (*i.e.* $160000 \times g$) for 60 h (20 °C) as previously described [28].

Polylysine binding

Polylysine was added to the nucleosome preparations in buffer B. In the respective experiments the amount of polylysine added is expressed as the molar ratio of lysine residues/nucleotide. In most cases described here the lysine/nucleotide ratio used was 0.3 since this represents the polylysine amount which would theoretically be sufficient to cover the linker DNA if it were the polylysine prime target site as initially anticipated (it has been shown [19] and will be discussed below, however, that polylysine appears to react readily with the nucleosomal core DNA, since at the lysine/nucleotide ratio given above cleavage between nucleosomes still occurs and the core DNA is protected against cleavage).

At polylysine concentrations above a lysine/nucleotide ratio of 0.5 aggregates are formed which are still accessible to internucleosomal cleavage but preclude the analysis by electron microscopy.

For electron microscopy nucleosome dimer samples were dialyzed against buffer C (10 mM sodium acetate, 5 mM triethanolamine, pH 7.9, 0.2 mM Na_2-EDTA [29]), polylysine was added at the respective lysine/nucleotide ratio and immediately thereafter the samples were fixed with glutaraldehyde as described below.

Ethidium bromide binding

Additions of ethidium bromide were also performed in buffer B (or in buffer C with samples to be analyzed by electron microscopy) and the dye concentrations are expressed as the ethidium bromide/nucleotide molar ratio. In most experiments the ratio chosen was 0.3 since we had demonstrated previously [30] that the cleavage pattern using micrococcal nuclease or DNase I was drastically altered under these conditions. Thus, this dye concentration should allow to compare the change in biochemical reactivity with the morphological appearance.

Nuclease cleavage and DNA determination

Cleavage with micrococcal nuclease was done in the presence of 1 mM $CaCl_2$ and 50 units of micrococcal nuclease/ml with samples containing 100 µg nucleosomal DNA/ml. The reaction was stopped after the time periods indicated with Na_2EDTA (final concentration: 2 mM) and by chilling in an ice bath.

After the nuclease cleavage experiments in the presence of ethidium bromide or polylysine the digestion mixtures were deproteinized in the presence of 1 M NaCl, 1% (w/v) sodium dodecyl sulfate and an equal volume of chloroform/isoamylalcohol (24:1) [31]. The aqueous supernatant was precipitated with 1.25 M perchloric acid, followed by a chemical DNA determination [32].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The cross-linked chromatin samples which had been dialyzed after exposure to formaldehyde extensively against double distilled water were lyophilized and dissolved thereafter in electrophoresis buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli [33]. The conditions of electrophoresis were the same as described earlier [28].

Electron microscopy

For electron microscopy, a modification of the BAC method [29] was used. Nucleosome dimer samples were adapted to an absorbance of 1.0 at 260 nm (*i. e.* 45 µg nucleosomal DNA/ml) and subsequently they were dialyzed against buffer C for 24 h at 4 °C. Then polylysine (lysine/nucleotide: 0.3 and 0.45) or ethidium bromide (dye/nucleotide: 0.3) were added or the samples remained untreated as controls. Immediately thereafter glutaraldehyde (final concentration: 0.1%, w/v) was added to the control and to the polylysine or ethidium bromide treated samples, respectively. The samples were kept at room temperature for 1 h and for additional 12 h in the cold. Finally, the solutions were diluted fiftyfold with buffer C and used for electron microscopy at the same day.

Before application to the grids aliquots of the samples were adjusted to 2×10^{-4} % (w/v) BAC and kept in an ice bath. After 1 min or after 20–30 min (with equal final results) droplets of 5 µl were placed onto carbon coated 400 mesh copper grids (freshly cleaned by glow-discharge) for 2–8 min in the cold. Then the grids were washed by floating (face down) on double distilled water for 10 min at room temperature, blotted with filter paper and air-dried. The grids were rotary-shadowed with tungsten-(VI)-oxide at an angle of 8–10 degrees in an Edwards vacuum coating unit E12E2 equipped with a liquid nitrogen trap. Electron micrographs were taken with a Siemens Elmiskop 101 at 80 kV and magnifications of 32000 and 80000.

Results and Discussion

Binding of polylysine to nucleosomal DNA

Polylysine binds to accessible phosphate groups of the chromosomal DNA. This binding is demonstrated biochemically in several ways. Titration curves have shown that aggregates are formed when half the DNA phosphate groups are covered with polylysine [18, 19].

Polylysine which is bound to the nucleosomal DNA can be cross-linked to its binding site using formaldehyde. This results in a decrease of the buoyant density of the polylysine/nucleosome cross-linked complex when compared with cross-linked authentic nucleosomes. The different nucleoprotein or nucleoprotein/polylysine complexes can be separated as cross-linked particles by cesium chloride equilibrium density centrifugation (Fig. 1). This apparent cross-linking of polylysine to the nucleosome suggests that its binding to the nucleosomal DNA occurs at accessible sites in a mode similar to the chromosomal proteins. No histones are released upon binding of polylysine [19] and both polylysine and histones remain associated with DNA in the high ionic strength cesium chloride gradient solution (when treated with formaldehyde). Electrophoresis of such cross-linked nucleosomes or polylysine/nucleosome particles on SDS polyacrylamide gels reveals a pattern of cross-linked histones which results from formaldehyde treatment. The control pattern of cross-linked products is in our system identical to the electrophoretic profile as published and identified by van Lente *et al.* [13]. In the presence of polylysine (lysine/nucleotide: 0.3) the pattern remains unchanged in the resolvable range of the electrophoresis gel. Thus, bearing the limitations of this approach in mind (only the oligomeric cross-linking products are seen) the data suggest that no changes of histone/histone contacts have arisen from the binding of additional (*i. e.* polylysine) molecules to the nucleosomal DNA (the electrophoretic patterns are not shown since they are identical to the data given in Fig. 4 for untreated and ethidium bromide treated particles).

A further effect of polylysine binding to nucleosomes is a decrease of the accessibility of the nucleo-

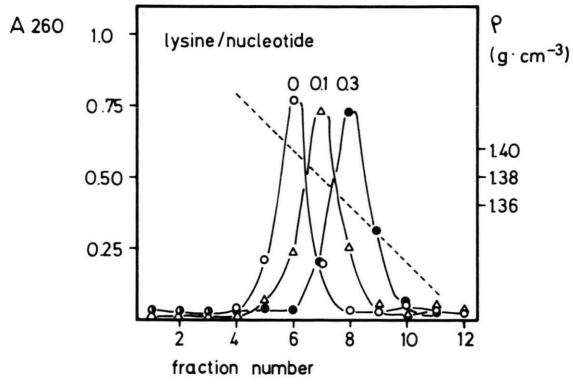

Fig. 1. Binding of polylysine to nucleosomes: cesium chloride equilibrium density centrifugation of nucleosome monomer/polylysine complexes after fixation with formaldehyde. The numbers on top of the gradient peak fractions represent molar ratios of lysine residues/nucleotide. Composite drawing of three separate cesium chloride gradients, the dashed line indicates the mean buoyant density profile of the three gradients. Similar CsCl gradient patterns were obtained with nucleosomal dimers after polylysine titration and formaldehyde fixation.

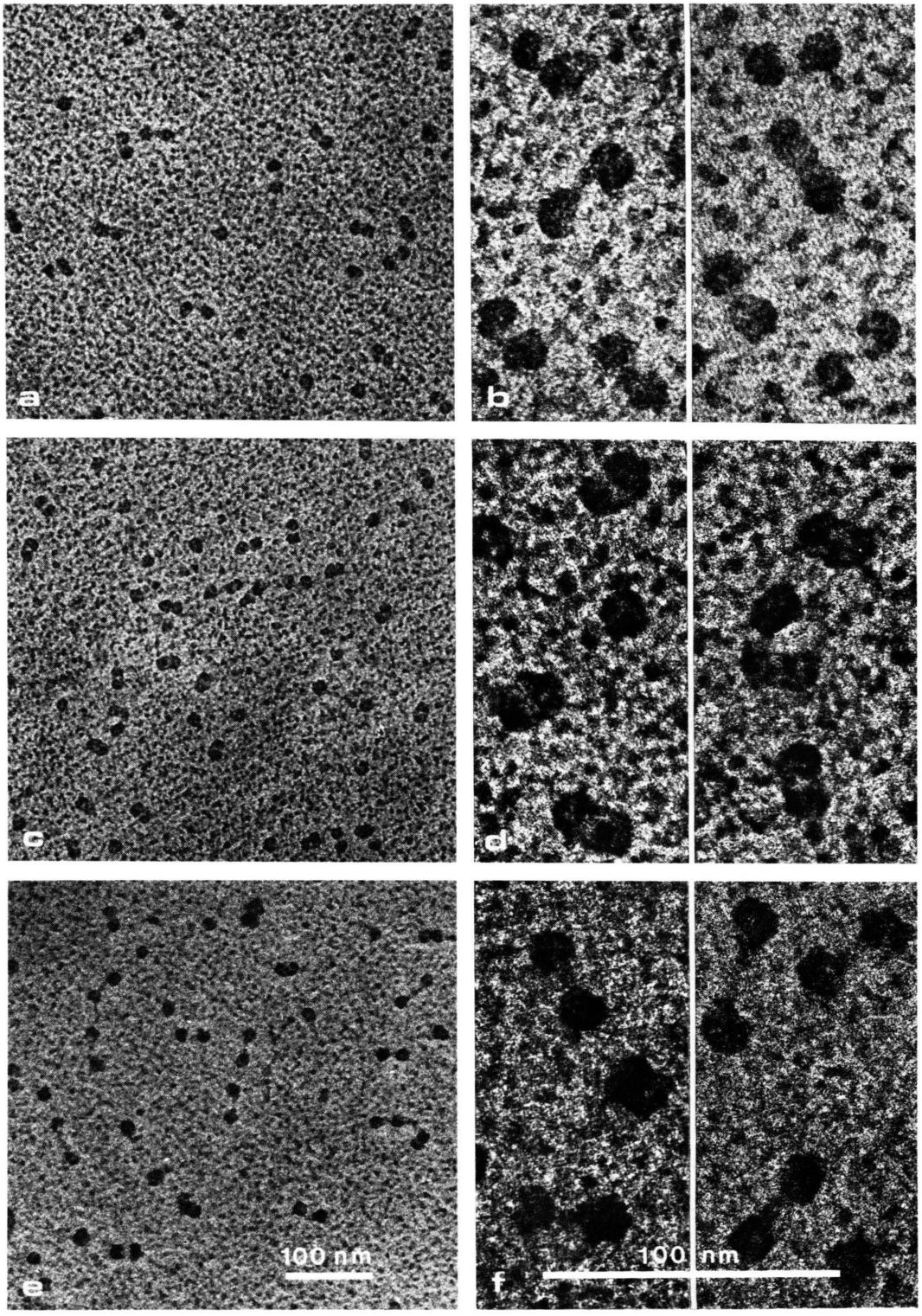

Fig. 2. Electron micrographs of nucleosome dimers from rat liver: a and b: untreated control preparation, c and d: treated with polylysine (lysine/nucleotide: 0.3), e and f: treated with ethidium bromide (dye/nucleotide: 0.3). Magnification: ×130 000 (a, c, e), ×450 000 (b, d, f).

somal DNA for cleavage with micrococcal nuclease. The degree of protection of the nucleosomal DNA is proportional to the amount of added polylysine (data not shown). This is in agreement with previous reports obtained with bulk chromatin preparations [18] which had been prepared by procedures involving mechanical shear which distorts the nucleosome structure [31].

We can conclude that polylysine at the lysine/nucleotide ratios investigated effectively interacts with the nucleosomal DNA since it can be cross-linked to the nucleosome and protects its DNA against degradation. On the other hand, as we have shown previously, cleavage between nucleosomes (*i. e.* at the linker DNA) is still possible even at a lysine/nucleotide ratio up to 0.7 [19] when the core DNA is inaccessible to nuclease attack. Thus, the initial cleavage site and the location of predominant polylysine binding do not coincide. Upon binding of polylysine the histone octamer is apparently not grossly altered, since no histones are released [19] and the pattern of histone oligomers resulting from chemical cross-linking remains unchanged upon polylysine treatment.

Electron microscopy of untreated nucleosome dimers

In the electron microscope the untreated nucleosome dimers (Fig. 2a and b) show the well-known beaded structure of two core particles connected by a fibrous strand which represents the linker DNA. After shadowing with tungsten oxide the projections of the particles appear as more or less round structures. The distances between the two cores of a dimer are not uniform (compare [34]) and the connecting fiber is not always clearly seen. The orientation of the two cores to their connecting strand seems to show center-to-center or zig-zag association as well as lateral attachment as it has also been described for the arrangement of nucleosomes in longer chromatin chains [35, 36]. Possibly, with nucleosome dimers these differences may just reflect different projections of the same structure. However, projections similar to the structures described below as predominating in polylysine or ethidium bromide treated samples, respectively, were only occasionally observed in the control preparations.

Electron microscopy of polylysine treated nucleosome dimers

After treatment with polylysine at a lysine/nucleotide ratio of 0.3 (Fig. 2c and d) the distance between the nucleosome cores is diminished and the two particles of a dimer seem to be in contact but they remain visible as two separate entities. Since it is difficult to trace the connecting fiber, it is not possible to decide whether its length is changed compared with the untreated samples.

Changes in size or shape of the cores apparently do not occur upon binding of polylysine though it appears that there is a preferential orientation of the two core particles towards each other within a dimer, possibly as a consequence of the distribution of polylysine accessible sites along the nucleosomal DNA. If the polylysine concentration was increased up to a lysine/nucleotide ratio of 0.45, similar results were obtained (micrographs not shown).

The effect of polylysine on the internucleosomal distance of the dimers is highly reproducible and the changes described here reflect a structural modification of the nucleosome dimers *per se*. The influence of a polylysine coating of the supporting film for an improvement of the adsorption of DNA as described by Williams [37] can be excluded here since in our system polylysine is entirely bound to the nucleosomal DNA and no free polylysine remains in the reaction medium [18, 19]. In addition, EDTA and surface active substances preclude this effect on the adsorption of DNA [37].

Comparison of biochemical and morphological effects of polylysine binding

In conclusion as to polylysine effects there is biochemical evidence that polylysine effectively binds to the nucleosomal core DNA instead of being confined to the linker DNA [19, 38, 39]. Since neither a lysine/nucleotide ratio of 0.3 nor of 0.45 leads to a morphological accentuation of the linker DNA (quite the contrary: it is difficult to trace the linker DNA), the biochemical data, which point towards an interaction between polylysine and the core DNA [19, 38] may thus be supported by the morphological appearance.

The distance between the nucleosomes is reduced upon polylysine binding but the presence of the polycation does not visibly affect the morphology of the nucleosome cores. A detection of an increase of the core particle size due to the association of polylysine with the DNA cannot be expected since the polylysine added amounts to less than 5% of the core particle mass. The unchanged morphology of the

core particles is, however, compatible with the tentative conclusions drawn from our cross-linking data that the internal nucleosomal core structure remains unchanged upon interaction of the nucleosomal DNA with polylysine at the lysine/nucleotide ratios tested. Thus, we conclude that increasing amounts of polylysine protect the nucleosomal core DNA (the binding to part of the linker DNA can, however, not be excluded) whereas the nucleosomal core structure apparently remains unaffected.

Binding of ethidium bromide to nucleosomal DNA

The binding of ethidium bromide induces changes in the structure of nucleosomes. This is documented in an altered accessibility of the nucleosomal DNA to micrococcal nuclease as well as to DNase I after addition of the dye [30]. After this enzymatic cleavage the DNA fragment pattern shows broadened DNA bands which indicates that the recognition of characteristic cleavage sites is less specific than in the absence of the dye.

In analogy to this qualitative assay the quantitative investigation of the ethidium bromide effect on enzymatic DNA cleavage in nucleosomes also reveals drastic changes upon addition of the dye (Fig. 3). The amount of nucleosomal DNA which is rendered accessible to micrococcal nuclease upon addition of ethidium bromide is increased but it reaches a plateau at 70% acid solubility in contrast to 50–60% in the absence of the intercalating dye. If the nucleosome structure would have been totally disintegrated in the presence of the dye, no such plateau should have been reached. Thus, we may conclude from this result that part of the nucleosome structure must have been preserved even in the presence of ethidium bromide.

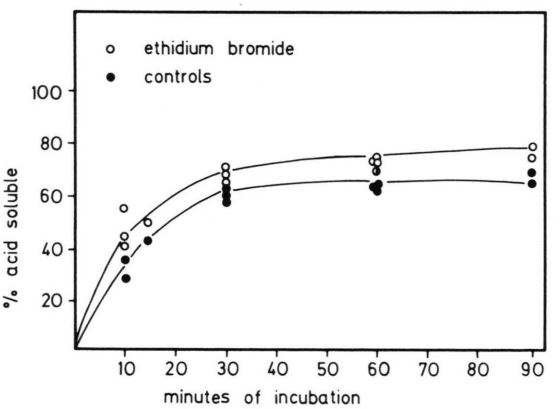

Fig. 3. Degradation of nucleosome monomers with micrococcal nuclease in the presence or in the absence of ethidium bromide: dye/nucleotide ratio: 0.3. The points are taken from four different experiments. Digestion conditions, deproteinization and DNA determination are described in the Materials and Methods section. Identical results as to the extent of digestion in the presence of ethidium bromide were obtained with nucleosomal monomers, dimers and trimers.

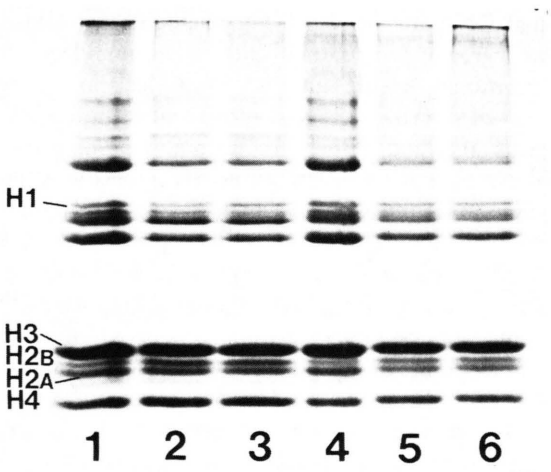

Fig. 4. Cross-linking of nucleosome monomers with formaldehyde under conditions of digestion with micrococcal nuclease: 1 ml samples of nucleosome monomers (100 µg/ml) were incubated with micrococcal nuclease (100 units/ml) in the presence of 1 mM $CaCl_2$ for 0, 5 and 10 minutes (lanes 1–3 and 4–6, respectively), then the material was chilled and fixed with formaldehyde after addition of Na_2-EDTA (final concentration: 2 mM). After HCOH fixation (1%, w/v) the material was dialyzed against water, lyophilized and analyzed by SDS polyacrylamide gel electrophoresis [33]. Lanes 1–3: control samples, lanes 4–6: digestion in the presence of ethidium bromide (dye/nucleotide: 0.3). The same cross-linking patterns were obtained with nucleosome dimers and trimers. The bands between H1 and H3 and the high molecular weight products have been described and identified by van Lente *et al.* [13] as cross-linked histones.

This is supported by the cross-linking data shown in Fig. 4. Addition of ethidium bromide to the nucleosomal preparation does not result in a rearrangement of the nucleosomal core histone moiety. This is shown by formaldehyde fixation of nucleosomes in the presence of ethidium bromide and subsequent analysis of the cross-linked particles (Fig. 4, slots 1 and 4). The patterns of oligomeric cross-linked histones in the dye-treated preparation appear indistinguishable from the cross-linked control.

This persistance of a histone/histone cross-linking pattern in the presence of ethidium bromide is also demonstrated in samples which were treated with micrococcal nuclease in the presence of the dye. For-

maldehyde fixation was done at different periods of time after the start of the digestion and a comparison with the digestion of the control preparation shows that the pattern of histones and histone oligomers remains unaffected during the cleavage process either in the absence or in the presence of ethidium bromide (Fig. 4).

Electron microscopy of ethidium bromide treated nucleosome dimers

After treatment with ethidium bromide the distance between the two core moieties of a nucleosome dimer is increased (Fig. 2e and f). The connecting fiber is elongated and more distinct compared with untreated samples. This apparent lengthening of the linker DNA can be explained by the stripping of part of the nucleosomal core DNA from the core histone octamer as described by Erard *et al.* [23]. Obvious changes in size and shape of the particles are not observed after addition of the dye. It seems, however, that the orientation of the connecting fiber towards the adjacent particles is less variable and appears stiffened in comparison with the untreated samples.

In the preparation shown here (dye/nucleotide: 0.3) not all particles show a reaction in terms of an increase of the distance between nucleosome cores. This is in agreement with observations obtained with nucleosome cores where 50% of the particles had shown a stripping of a terminal part of the nucleosomal core DNA from the histone octamer when treated with the dye [23].

Comparison of biochemical and morphological effects of ethidium bromide binding

In conclusion we have found that addition of ethidium bromide to chromatin causes a structural alteration which can even be observed with nucleosomal dimers as a model preparation. The morphology of the core particle is not altered and the histone arrangement within the core appears to be unchanged in the presence of the dye, but the distance between the two cores of the nucleosome dimer and the length of the connecting fiber are increased. Concomitantly, the rate of degradation by micrococcal nuclease increases but it reaches a plateau level. Even during this degradation (which shows a loss of specificity as to accessible cleavage sites) the arrangement of core histones appears to remain unaffected in the presence of the dye.

Conclusions

Polylysine interacts with the nucleosomal DNA and protects the core DNA against enzymatic degradation. According to model studies of Mirzabekov and Rich [40] the mode of interaction of histones and DNA in nucleosome cores implies an exposure of part of the DNA phosphate groups outside. The distribution of such histone free gaps on the DNA of the dynamic nucleosome structure has been studied in detail by Shick *et al.* [17]. The authors discuss that these sites including the first 20 nucleotides from the 5′-ends of the core DNA (which are uncovered by histones, too) should be easily accessible to molecules such as histone H 1 or polylysine [17].

A further argument in favour of a binding of polylysine to accessible sites along the nucleosomal core DNA is the observation that the histone/DNA ratio per nucleosome core can be higher than the histone octamer/146 base pair ratio [41].

Several modes of binding of polylysine to nucleosome dimers (as investigated here) would be compatible with the morphological data. The polyanion could bind to accessible sites of individual cores of a dimer or it could bridge the space between both core moieties of a dimer attaching these to each other. A further possibility would be a linking of several nucleosome dimers to each other, resulting in the formation of aggregates. This, however, occurs only at higher polylysine concentrations and renders the electron microscopical investigation difficult.

Our biochemical results obtained with ethidium bromide also agree with the ultrastructural data. The dye-induced changes render an increased amount of nucleosomal DNA accessible to micrococcal nuclease and reach a plateau of digestion at a smaller DNA fragment size than the controls [25, 30, 39]. This indicates that the remaining nucleosome core structure still protects part of the nucleosomal DNA. Since the cross-linking pattern of histones is unchanged even during the course of digestion in the presence of the dye we conclude that the alteration of the nucleosome structure is restricted to the histone/DNA interaction without affecting the internal histone octamer arrangement. This results in a partial release of DNA from its close contact with the histone octamer which leads to a lengthening of the linking DNA fiber between core particles at the expense of the core DNA.

This stripping of the terminal core DNA from the histone octamer, which has first been described by Erard *et al.* [23] and explains our results with nucleosome dimers (Fig. 3e and f), might be facilitated by the fact that the first 20 nucleotides from the 5′-ends of the core DNA are not covered by histones [17].

The binding of ethidium bromide occurs electrostatically at high affinity sites to the linker DNA [23, 25] and the dye interacts in a cooperative intercalation process with the core DNA stripping part of it from the histone octamer. Alternative explanations for the lengthening of the linker DNA, such as an increase in length due to the intercalation of the dye [42, 43] are excluded by the electrostatic mode of binding (which has also been described *in situ* [44]) of ethidium bromide at the linker DNA [25].

Recently, Wu *et al.* [45] have derived from fluorescence polarization and electric dichroism measurements a model which involves the conversion of the nucleosome core into a more extended structure and requires protein/protein movements upon intercalation of the dye. The increase in linker DNA length described here may reflect morphologically the postulated extension of the nucleosome structure. We could, however, not observe drastic changes of histone/histone contacts.

In conclusion, we have shown that both the interaction of polylysine and of ethidium bromide with the nucleosomal DNA induce structural alterations. These influence biochemical parameters and change the distance and orientation of nucleosomal core particles towards each other when dimers are used as a model preparation.

Acknowledgements

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