Proximity and accessibility studies of histones in nuclei and free nucleosomes

William M. Bonner

Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014, USA

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ABSTRACT

Histone proximity in chromatin was studied with the cleavable crosslinking reagent, dithiobissuccinimidyl propionate. Crosslinks between H4 and H2a, H4 and H2b, H4 and H3, H2a and H2b, H2b and H3 were found. H1 is also crosslinked to the nucleosomal histones.

In nuclei, unsheared chromatin, and H1 depleted chromatin, the four nucleosomal histones are crosslinked at similar relative rates both in 5 mM salt and 100 mM salt. After micrococcal nuclease treatment to generate nucleosomes, H2a and H2b are crosslinked faster than H4 and H3. C^{14} -NEM titration of thiopropionate residues bound to each histone shows that H2a and H2b are more accessible to this reagent after nuclease treatment but that the increased binding was not sufficient by itself to explain the increase in crosslinking.

Bolton Hunter reagent was used to further study the accessibility of the four nucleosomal histones in whole chromatin and nuclease digested chromatin. These studies showed that salt increases the accessibility of all four histones while nuclease treatment decreases H4 accessibility.

INTRODUCTION

Histones in chromosomes are not distributed evenly along the DNA, but in bodies called v bodies or nucleosomes (1-10), containing histones H2a, H2b, H3 and H4 with about 140 base pairs of DNA (11-12). The arrangement of the histones in the nucleosomes has been the subject of many investigations. One useful tool has been protein crosslinking reagents (13-20). We have used the cleavable crosslinking reagent dithiobissuccinimidyl propionate to crosslink whole chromatin and nuclease digested chromatin to compare the histone conformations in these two structures.

Our results show that although nuclease treatment does not alter the type of crosslinks formed between histones, it does change the relative rates of crosslinking. In nuclei or whole chromatin the four nucleosomal histones are crosslinked at similar rates. After nuclease digestion the

crosslinking of H2a and H2b is accelerated while the crosslinking of H3 and H4 remains unchanged. These results can be partially correlated with changes in histone accessibility in the two structures.

MATERIALS AND METHODS

Dithiobissuccinimidyl propionate (SP) was from Pierce Chemical Co. Staphylococcal nuclease was from Worthington. I^{125} Bolton Hunter and C^{14} -NEM reagent was from New England Nuclear. Sequenced African Green Monkey satellite segments were a gift from M. Rosenberg.

Rat liver nuclei were prepared as previously described (21). Nuclei at 1 mg DNA/ml were treated with 10 μ g staphylococcal nuclease/ml for various times at 37°C in a buffer containing 5 mM triethanolamine (TEA) HCl pH 8.0, 0.5 mM $CaCl_2$. Na_2EDTA was added to a concentration of 1 mM to stop the reaction. Undigested nuclei received Na_2EDTA before the enzyme. Aliquots were frozen for analysis of the DNA on 6% acrylamide gels using Loening's buffer (22). The size of the DNA was measured relative to sequenced Green Monkey satellite pieces 172 bp and 344 bp long (23).

Untreated or nuclease treated nuclei were crosslinked routinely with 0.5 mg SP/ml in the above buffer. The presence or absence of staphylococcal nuclease in the undigested nuclei had no effect on the extent or type of crosslinks formed. Reaction mixtures were incubated for times up to 1 hour at 22°C. To stop the reaction, mixtures were made 2% in SDS and 5 mM in NEM, and incubated for another 15 minutes at 22°C. The reaction was then made 0.06 M in Tris HCl buffer pH 6.8 and either frozen at -20°C or immediately electrophoresed on an 15% SDS-polyacrylamide slab gel (24). Gels, 27 cm long, 15 cm wide and 1.5 mm thick, were electrophoresed for 18 hrs at 15 ma, then stained in 0.1% Coomassie Blue R, 40% ethanol and destained in 5% acetic acid, 10% ethanol.

For two dimensional gel electrophoresis, the second gel was 27 cm long, 15 cm wide and 2.25 mm thick, and the stacking gel had no slots. Strips one to two cm wide containing the bands to be analyzed were cut from the first dimension gel, soaked for two hours in 0.06 M Tris HCl pH 6.8, 5% mercaptoethanol, and laid on top of the stacking gel for the second dimension. Electrophoresis in the second dimension was for 18 hours at 20 ma. These gels were stained and destained as the first dimension gels.

The binding of SP to histones was measured by reducing crosslinked nuclei with 10 mM dithiothreitol, dialyzing the nuclei vs 10 mM KP buffer pH 7.0 and reacting the free thiol groups with 1 mM C^{14} -NEM for 15

minutes at 22°C. The reaction was stopped with SDS gel sample buffer (24) and the samples were electrophoresed in 15% SDS acrylamide gels. After fluorography (25), the dried gel was sliced, and the slices were digested with 95 parts 30% H_2O_2 -5 parts concentrated NH_4OH . These samples were then counted in Aquasol.

Lysed nuclei were reacted with Bolton-Hunter reagent under the same conditions as with crosslinking reagent. Samples were analyzed by gel electrophoresis as described above for C^{14} , except that fluorography was not necessary with I^{125} . The gel slices were counted in a gamma counter.

RESULTS

Controls. When chromatin or nuclei are reacted with dithiobissuccinimidyl propionate (SP), a crosslinked structure results in which proteins are linked to each other through disulfide bridges. Since preexisting disulfide bridges through cysteines in the uncrosslinked protein would also contribute to the final crosslinking pattern, it is necessary to determine if any crosslinks in untreated chromatin are present.

When uncrosslinked chromatin prepared without NEM was subjected to two dimensional gel electrophoresis, H3 dimers were found (Figure 1c); however, NEM was found to prevent H3 dimer formation (Figure 1b). Further study showed that NEM prevented H3 dimer formation if it was present during denaturation of the chromatin (Figure 1d). If NEM was present only while chromatin was native but removed before denaturation, H3 dimers were still found (Figure 1a. The same effect was also seen with crosslinked chromatin (data not presented). The crosslinking pattern contained $(\text{H3})_2$ when NEM was not used, but lacked $(\text{H3})_2$ when chromatin was denatured in the presence of NEM. For this reason chromatin was routinely denatured in the presence of NEM.

The effect of the crosslinker on the integrity of chromatin was also studied (Figure 2). Staphylococcal nuclease digested crosslinked chromatin to nucleosomes at a rate similar to the digestion rate of uncrosslinked chromatin. This result indicates that the chromatin has not been denatured by exposure to the crosslinking reagent.

Crosslinks. When chromatin is crosslinked as described in Methods, a number of new broad bands appear at R_f 's corresponding to multiples of the nucleosomal histones while most of the nonhistone proteins are crosslinked to large structures which do not enter the gel. When this gel is reduced and electrophoresed in the second dimension, a two dimensional array of spots result (Figures 3 and 4).

FIGURE 1: Formation of $(H3)_2$ under various conditions. Lysed nuclei were prepared from rat livers as described in Methods either with (a, b) or without (c, d) 5 mM NEM. NEM was removed by dialysis. Half of each sample was then incubated in 2% SDS with 5 mM NEM, (b, d) the other half (a, c) without NEM. The samples were subjected to two dimensional gel analysis as described in Methods. Only the H1 region of the first dimension gel was subjected to two dimensional analysis, since this is where $(H3)_2$ migrate. No other non diagonal spots were found on the gels.

- a) Exposed to NEM before denaturation only.
- b) Exposed to NEM before and after denaturation.
- c) No NEM
- d) Exposed to NEM after denaturation only.

This low resolution gel shows the general characteristics of chromatin crosslinking. Regions II, III, and IV are found to contain all the nucleosomal histones. At this resolution, the only visible differentiation is that the H4 spot in regions II and III is slightly to the left of the other histones. In addition to the nucleosomal histones, regions III and IV also contain H1a and H1b. Their presence in region III indicates that considerable crosslinking between H1 and nucleosomal histones has taken place, since this is where an H1-(nucleosomal histone) dimer would migrate.

Higher resolution gels of the dimer region (Figure 5 and 6) clearly indicate several of the crosslinks formed. H4 is linked to H2a, H2b, and H3. H4 from these three dimers has presumably fused into one band during electrophoresis. At a slightly higher molecular weight H2a-H2b and H2b-H3 dimers are found.

FIGURE 2: Micrococcal nuclease digestion patterns of DNA in control and crosslinked chromatin. Chromatin was crosslinked as described in Methods, and dialyzed vs 5 mM TEA pH 8 to remove excess reagent. Aliquots of chromatin containing 1 mg/ml DNA were made 0.1 mM CaCl_2 and 10 $\mu\text{g/ml}$ micrococcal nuclease. After incubation at 37°C for various times, the reaction was terminated by adjusting the solution to 20 mM Na_2EDTA pH 7, 0.1% SDS and 1 M NaCl. The aliquots were extracted with buffer saturated phenol, and the DNA was precipitated with two volumes of 95% ethanol at -20°C . Samples were electrophoresed on 5% acrylamide gels with Loening buffer (22). Left column, control. Right column, crosslinked. From top to bottom, 0, 1, 2, 5, 10 minutes digestion.

Similar treatment of the trimer region (Figure 7 and 8) resolves the crosslinks only into a set of bands containing H4 and a set lacking H4. H1 is also found crosslinked in this region. Since the crosslink molecular weight is close to 1.5 times the molecular weight of H1, these crosslinks are presumably between H1 and nucleosomal histones. H1a divides into two spots, one of which is above H4 and therefore a presumptive H1-H4 link. The other major spot is H1a linked to some or all of the other nucleosomal histones. H1b is also crosslinked to the nucleosomal histones.

Kinetics of Crosslinking. During reaction of lysed nuclei with SP, the four nucleosomal histones disappear at similar rates. However, digestion of the lysed nuclei with staphylococcal nuclease increases the rate of disappearance of H2a and H2b but does not affect the rate of disappearance of H3 and H4.

Figure 9 shows that after five minutes nuclease treatment, H2a and H2b are 50 to 60% crosslinked compared to 25 to 30% without nuclease

FIGURE 3: Two dimensional gel of the whole first dimension gel. All procedures are described in Methods.

FIGURE 4: Diagram of Figure 3.

FIGURE 5: Two dimensional gel of region II of Figure 4. All procedures are described in Methods.

FIGURE 6: Diagram of Figure 5.

treatment. H4 is crosslinked to the same extent with and without nuclease treatment while H3 is somewhat less crosslinked. After 5 minutes nuclease treatment, the DNA is predominately in monomers with some dimers, trimers and tetramers present. No bands smaller than monomers are seen.

Results after one minute nuclease treatment are intermediate between

FIGURE 7: Second dimension gel of region III of Figure 4. All procedures described in Methods.

FIGURE 8: Diagram of Figure 7.

those after no treatment and 5 minutes treatment. The DNA after one minute nuclease treatment is about 30% in monomers with most larger than trimers. After one minute nuclease treatment, the crosslinking of H2a and H2b has increased to 40-45%, while the crosslinking of H3 and H4 is relatively unaltered.

The size of the DNA was measured against sequenced DNA fragments. No material smaller than 150 bp was present at one minute, while at five minutes a leading shoulder at 135 bp was present in small amounts. With 10 minutes SP crosslinking (Figure 9), the one minute nuclease digestion accounts for most of the increased crosslinking of H2a and H2b. These results argue against the involvement of internal nucleosomal DNA breaks in the increase of H2a and H2b crosslinking.

The pattern of histone dimers, trimers, and tetramers is similar with and without nuclease treatment except that trimers and tetramers and to a slight extent some dimers are increased after nuclease treatment, due to the increased crosslinking of H2a and H2b. No differences is seen in the kind of crosslinks found on two dimensional gels (data not presented).

Since nuclease digestion of the spacer DNA may lead to the removal of H1 and most nonhistone proteins from the chromatin, the effect of removing these proteins on the crosslinking rates was investigated. Removal of H1 and the nonhistone proteins soluble in 0.7M NaCl does not alter the relative extents of crosslinking of the nucleosomal histones. The results suggest that only the nucleosomal histones and DNA are involved in the effect. In addition, the relative extents of crosslinking of the nucleosomal histones are not changed in whole nuclei as compared to lysed nuclei (data not presented).

The effect of ionic strength on the crosslinking was studied for two reasons. Firstly, increased ionic strength should increase the extent of crosslinking by loosening the association of histone basic residues for DNA. Secondly, increased ionic strength may affect histone H2a and H2b differently than H3 and H4 because of their different lysine and arginine contents. 100 mM NaCl does lead to greater crosslinking (about 80% compared to 20% without NaCl,) but without nuclease treatment, the histones still disappear at similar rates. After 5 minute nuclease treatment, H2a and H2b are still crosslinked faster relative to H3 and H4 in 100 mM NaCl. After 1 hour M SP crosslinking all the H2a and H2b is crosslinked.

Binding of Crosslinker. Since crosslinking may not be very efficient in terms of crosslinks formed per molecule bound, nuclease treatment could

increase the crosslinking of H2a and H2b without increasing the binding of crosslinker just by increasing the efficiency of crosslinking, perhaps by removing some spacer DNA that hindered crosslinking but not binding. To test this, the thiopropionate groups bound to the histones were titrated with C^{14} -NEM. Since under these conditions, twice as much H2a and H2b

FIGURE 9: Time course of histone crosslinking with and without nuclease treatment.

Chromatin was nuclease treated and crosslinked as described in Methods. At various times, aliquots were quenched with 2% SDS, 5 mM NEM, 0.05 M Tris HCl pH 6.8 and electrophoresied on SDS gels. The gels were stained, and photographed. The density of stain was measured from the negatives with a Joyce-Lobel densitometer. Peak heights were measured from the densitometer tracings and plotted as percent of the uncrosslinked control vs time of crosslinking.

- No nuclease treatment
- 1 minute nuclease treatment
- 5 minute nuclease treatment

are crosslinked after nuclease treatment (Figure 9), one might expect twice as much SP to be bound if crosslinking efficiency is to be unchanged. At the other extreme, an increase in crosslinking efficiency could take place with no increase or even a decrease in bound SP.

Figure 10 shows that intermediate results are obtained for H2a and H2b.

About 25% more crosslinker is bound to H2a and H2b after nuclease treatment. Since these results fall considerably short of the expected 100% increase if crosslinking efficiency remained the same, they do strongly suggest that the efficiency of crosslinking has increased.

The arginine rich histones also bind similar amounts of SP as H2a and H2b. H4 is relatively unaffected by nuclease treatment with regard to both crosslinking (Figure 9) and binding (Figure 10). With H3 the results are seemingly paradoxical. H3 does bind more crosslinker after nuclease treatment, even though crosslinking is not increased and actually slightly decreased. This means that although more SP was bound, the crosslinking efficiency of H3 bound SP was decreased.

In order to gain more insight into the availability of histone residues to chemical probes, chromatin was reacted with I^{125} Bolton-Hunter reagent under various conditions. Bolton Hunter reagent is a

FIGURE 10: NEM titration of thiopropionate groups bound to histones. Procedures are described in Methods. The amount of NEM bound to H4 was calculated to be approximately 0.9 moles NEM/mole H4. Somewhat more is bound to the others.

succinimidyl ester like SP and therefore might be expected to react similarly but its binding can be measured directly. Figure 11 shows that Bolton Hunter reagent shows the same effect as does SP (as titrated with C^{14} -NEM); nuclease treatment leads to a decrease in H4 reactivity and increases in the others. The changes are also similar in magnitude to the changes seen with C^{14} -NEM.

The relative effect of ionic strength is seen clearly. 50 mM NaCl leads to more than two fold increases in H2a, H2b, and H3 reactivities and 35% increase in H4 reactivity. Nuclease treatment causes a 25% decrease in H4 reactivity and 20-50% increases in the reactivity of the others. It is interesting to note that the effect of nuclease treatment is salt independent even though the amount of binding of I^{125} Bolton-Hunter reagent is salt dependent. These results parallel those obtained above concerning the amount of SP bound to the histones with and without nuclease treatment.

Bolton-Hunter reagent and SP are thought to bind primarily to lysine amino groups. The DNA digested by nuclease may have freed some lysine amino groups from ionic bonds and this may account for the increased binding of SP and Bolton-Hunter reagent to H2a, H2b and H3 after nuclease treatment. For this reason, histone lysine availability was tested by acetylating chromatin under competing conditions (26). Under these conditions in whole chromatin only the amino terminal of H2b reacts as a free amino group; H4, H2a and H3 do not incorporate significant amounts of acetate. Nuclease digestion of the chromatin does not alter this result indicating that no lysine amino group is acetylated as free lysine either before or after

FIGURE 11: Reaction of Bolton Hunter Reagent with nucleosomal histones under various conditions. All procedures are described in Methods.
 A) 5 mM TEA buffer pH 8.0 — no nuclease — — — nuclease.
 B) 5 mM TEA, 50 mM NaCl — no nuclease — — — nuclease.

nuclease treatment (data not presented). Therefore although nuclease treatment makes H2a, H2b and H3 more available to SP and Bolton-Hunter reagents, none of the lysine amino groups can be acetylated as free amino groups.

DISCUSSION

SP forms the crosslinks H4-H2a, H4-H2b, H4-H3, and H2a-H2b and H3-H2b. Crosslinks between H1 and the nucleosomal histones were found, a putative H1-H4 being resolved.

(H3)₂ were not seen when chromatin was denatured in the presence of NEM. In this respect our results differ from earlier work with cleavable crosslinkers. It is interesting to note that (H3)₂ have been reported only with cleavable crosslinkers (19, 27) containing a disulfide bond. With these crosslinkers, reducing agents cannot be used while the crosslinked histones are removed for gel analysis, either with SDS or acid. Blocking the sulfhydryls while the chromatin is native is ineffective because the H3 sulfhydryls are buried (data not presented). Upon denaturation, these sulfhydryls are exposed, allowing H3 dimers to form. These results indicate that reports of (H3)₂ in chromatin crosslinked with cleavable crosslinkers should be treated with caution unless the relevant controls are reported.

Most of the crosslinks seen in this report agree with those seen by other workers with cleavable crosslinkers. The major difference is that we see H3-H2b but no H3-H3, while others find H3-H3 (19, 27) and in one case no H3-H2b (27). These differences could be due partly to differences in conditions and reagents but also possibly due to the need to block H3 sulfhydryls as discussed above.

When succinimidyl propionate crosslinks two molecules a 12^oÅ spacer composed of 6 carbons and 2 sulfurs is interposed between the two molecules. Some other studies have identified histones crosslinked with spacerless crosslinkers. This laboratory has reported that carbodiimide can condense histones H3 and H4 (13). Carbodiimide also forms H3-H2b and H3-H2a crosslinks (Bonner, unpublished results). UV light crosslinks H2a and H2b (15), while tetranitromethane crosslinks H4 and H2b (14). Formaldehyde, a crosslinker with a one carbon spacer, also forms H2a-H2b and H4-H2b (16).

Succinimidyl propionate forms all these crosslinks plus some of H4-H2a. The small amount of H4-H2a plus the fact that it is not seen with spacerless crosslinkers may indicate that these two histones are not as

closely associated as the others.

When nuclei, lysed nuclei, or nuclei extracted with 0.7M NaCl are crosslinked with SP the nucleosomal histones are crosslinked at similar rates. After treatment with staphylococcal nuclease, more H2a and H2b is crosslinked under the same conditions.

Thomas and Kornberg (17) reported that dimethylsuberimide crosslinks H2a and H2b faster than H3 and H4 in partially digested chromatin. Because their chromatin was digested to weight average 1600 base pairs, it is not possible to compare their results directly with these.

Hardison et al. (19) also found differences in crosslinking rates with methyl-4-mercaptobutyrimide. Neither group looked for differences between undigested chromatin and nucleosomes. Weintraub et al. (28) reported that Staphylococcal nuclease digestion did not alter the rate of histone digestion by trypsin.

H2a and H2b have more lysine and less arginine than H3 and H4, and are removed from the nucleosome by 1.3M NaCl, while 2M NaCl is necessary to remove H3 and H4. Therefore it is possible that the nuclease effect could be mimicked by increased salt. However, increased salt leads to proportional increases in the crosslinking of all four nucleosomal histones and also an increase in binding Bolton Hunter reagent. This effect is qualitatively different from the nuclease effect. Therefore nuclease treatment is affecting histone accessibility differently than salt is.

Since nuclei, lysed nuclei, and 0.7M NaCl extracted nuclei are crosslinked with similar relative kinetics, histone H1 and most nonhistone proteins do not seem to participate in the effect. Also the difference in compaction of chromatin seen in nuclei vs lysed nuclei does not affect the relative kinetics of crosslinking under these conditions.

These findings suggest that this effect is primarily due to the digestion of non-nucleosomal DNA. The decreased reactivity of H4 coupled with the increased reactivity of H2a, H2b and H3 indicate that a conformation change has occurred upon digestion. The result can not easily be explained by a loosened DNA protein binding at the ends of the nucleosomal DNA because the evidence indicates that H2a and H2b are not associated with the ends of the nucleosomal DNA (29).

There are two models which may be proposed to explain these findings. In the first model, a binding site for spacer DNA exists on the nucleosome. Digestion of this DNA leads to a conformational change. In the second

model, the spacer DNA does not bind to the nucleosome but does maintain nucleosomes juxtaposed. Digestion of spacer DNA allows the nucleosomes to separate and undergo conformational change. These two models are currently being tested.

Abbreviations: SP - dithiobissuccinimidyl propionate; NEM - N-ethylmaleimide; TEA; - triethanolamine; KP - potassium phosphate.

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