Cross-linking of nucleosomal histones with monofunctional imidoesters

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ABSTRACT

Cross-linking experiments with the MONOfunctional imidoester methyl-acetimidate, in the pH range 7.0 - 8.0, on rat liver nucleosomes generate a cross-linking pattern almost identical with the one observed for much longer Bifunctional reagents (e.g. dimethylsuberimidate).

Combined cross-linking and trypsin digestion experiments suggest that all or at least the great majority of this cross-linking occurs on trypsin digestible segments (or "tails") of the histones.

The formation of oligomers over such extremely short distances and especially the observation of an H3 homodimer suggests a very close proximity of half-nucleosomes.

INTRODUCTION

Cross-linking with bifunctional imidoesters was one of the approaches originally leading to the nucleosome concept (1). Discrete oligomer patterns thus generated and observed in SDS electrophoresis give information on the relative location of histones in a nucleosome particle (2). The major part of this work was performed with bis-imidates containing a $-(CH_2)_6-$, $-(CH_2)_4-$ or a cleavable $-(CH_2)_2-S-S-(CH_2)_2-(3,4)$ linker with a separation (in a fully extended reagent) of approximately 11 Å (and 9 Å for $-(CH_2)_4-$) between the reactive groups.

Careful inspection of the reaction mechanism of amidine formation (5) also indicates the cross-linking potentiality of monofunctional imidoester moieties by formation of bisubstituted amidines. This feature and the awareness of the mildness and specificity of amidination (6) resulted in an analysis of the cross-linking capacity of methyl-acetimidate ($CH_3C(=NH)OCH_3$) on rat liver nucleosomes.

MATERIALS AND METHODS

Reagents. Methylacetimidate hydrochloride, dimethylsuberimidate dihydrochloride and dimethyladipimate dihydrochloride (abbreviated as MAI, DSI, and DAI respectively) were purchased from Pierce. Infrared

spectra show characteristic 3310 cm^{-1} (N-H) and 1650 cm^{-1} (C=N) absorption bands and nitrile, ester or amide II bands were not detectable.

MAI was also synthesized according to the Pinner synthesis (7) and showed an IR spectrum identical to the purchased pure material. Lysozyme was grade I Sigma.

Histone preparations. H2A-H2B complexes and (H3H4)₂ tetramers were isolated according to the method of Van der Westhuyzen et al. (8). The pH in the G50 and G100 columns was at 6.0 and 0.01M EDTA was added to retard pH changes resulting from HSO_3^- (9) decomposition. Individual histones were obtained from biogel P60 chromatography in 0.02M HCl, 0.1M NaCl of whole histone, H2A-H2B or (H3H4)₂ complexes (10). All preparations were tested for purity by SDS polyacrylamide electrophoresis.

Nucleosome preparations and characterization. Nucleosomes were prepared by in situ digestion of rat liver nuclei with micrococcal nuclease (Worthington) as described by Noll (11). The digest was fractionated by zonal ultracentrifugation at 4°C on a 490 ml, 7%-38% hyperbolic sucrose gradient in 10 mM Tris-HCl, 1 mM EDTA, 10 mM NaHSO_3 , pH 7.5, in a Ti 14 Beckman rotor.

Nucleosomes were prepared in a digestion buffer containing no spermine or spermidine (10 mM Tris-HCl, 2 mM CaCl_2 , 0.25M sucrose, pH 8.0).

Monosomes from the zonal run were pooled and showed up as 10.9-11.4 $S_{20,w}$ particles on linear 6%-30% sucrose gradient. The CD spectrum of the nucleosome DNA was as described by Lilley (12). Monosome DNA runs as a homogeneous band as well on non-denaturing as denaturing 5% polyacrylamide gels (13).

Histone was checked for proteolysis on 17.5% SDS-polyacrylamide gels. Material was stored in 10 mM Tris, 1 mM EDTA, 10 mM NaHSO_3 , 1 mM PMSF, pH 7.4 at -70°C.

Cross-linking procedure. Reactions were performed at 3 mg/ml of cross-linker, 20°C for the indicated times (see figures). Buffers mostly used were phosphate 70 mM, pH 8.0, (ionic strength= 0.2). Other buffer systems are indicated in the text and under figures. Reagents were dissolved, brought very quickly to the desired pH and added to the reaction mixture. The pH was kept at constant value with diluted HCl.

Ammonolysis and dimer processing. Dimer bands were cut from SDS gels after a light coloration with Coomassie Blue R-250 and short rinsing in 7.5% Acetic acid as described by Goff (14). Eluted material was precipitated with acetone and ammonolysed as described by Hunter

(15) with ammonia-acetic acid (15/1; v/v), pH 11.3 for 50 hours at room temperature. Afterwards it is dialysed against water, lyophilized and rerun on gels.

Autoradiography of reconstituted materials. Radioactive methylation was performed on biogel purified histones, H2A and H2B, as described by Martinson (16) : reductive methylation (17) with HC^{14}HO (NEN, 10 mCi/mMol).

Reconstitution was performed according to one of the procedures described by Felsenfeld (18) and in which step-dialysis starts from 5 M Urea-2 M NaCl. Calf thymus DNA (Miles) was phenol-treated and sonicated before use.

Prior to autoradiography, gels were left for one night in 50% methanol, 3% glycerol in H_2O and subsequently dried.

SDS electrophoresis. 17.5% polyacrylamide sodium dodecyl sulphate gels, 20 cm long and 0.15 cm thick, were made using a modification of the method of Laemmli (19). The separating gel has an acrylamide/bis-acrylamide ratio of 525/2.2 in 0.33 M Tris-HCl pH 8.7, 0.1% SDS. The stacking gel is 5% acrylamide with an acrylamide/bisacrylamide ratio of 100/5 in 0.1% SDS, 0.125M Tris pH 7.8. Electrophoresis is run at 150V, using a 0.05 M Tris, 0.38 M Glycine, 0.1% SDS buffer. Samples are dissolved in 0.1 M DTT, 4% SDS, 0.08 M Tris pH 7.8, 10% (v/v) glycerol and boiled for 1 min. prior to gel loading. (Nucleosomes are also treated this way, without prior histone extraction).

Gels were stained with Coomassie Blue G 250 (Polyscience) perchloric acid (20).

Trypsin (Trypsin TPCK - Worthington) digestions were performed at pH 7.4 in 1 mM EDTA solutions at 10 $\mu\text{g/ml}$ at 37°C for 2 hours. Carbon¹⁴ counting was performed in unisolve-1 (Koch-Light) at 1/10 H_2O containing sample/scintillation liquid volume ratios.

RESULTS AND DISCUSSION

Comparison of cross-linking patterns with DAI, DSI and MAI. We have focused special attention on the dimer patterns. The different dimers are clearly resolved and very characteristic (Fig.1). With DAI and DSI three bands are always observed in our 20 cm long gels when reactions are performed in buffers at pH 8.0 ($I=0.2$) and pH 9.0 ($I=0.1$) and these can be identified with those described by Thomas and Kornberg for DSI (3).

The same symbols as in reference (3) were attributed to these bands

Figure 1. Cross-linking patterns on 17.5% SDS-polyacrylamide gel of nucleosomal histones with (A) dimethylsuberimide (B) methylacetimidate. Reactions were performed in 70 mM phosphate buffer (pH 8.0; $I=0.2$). Imidoesters used at 3 mg/ml. Nucleosome concentration: 0.2 mg/ml nucleosome ($O.D._{260}=2.0$).

(I, II, III, see figures) and a comparison of slots a and d in figure 5 shows that band I migrates very slightly beyond H1 as described in this same paper.

With MAI in 70 mM phosphate pH 8 ($I=0.2$) buffers a very similar dimer pattern is generated. Products I', II', III' migrate at identical positions as I, II, and III, but in addition a fourth sharp band (Ia) is always observed.

Identical cross-linking patterns are also obtained in 10 mM phosphate buffers (pH 8.0), at 4°C instead of 20°C and with nucleosomes prepared without spermine or spermidine (see materials and methods), so that any residual amines of this sort, which might remain firmly bound to the particles, are not responsible for the features of the pattern. In most experiments we used the 70 mM (pH 8.0) phosphate buffers to allow an accurate comparison with the afore-mentioned references.

The cross-linkings observed are intranucleosomal. In order to prove this point, nucleosomes from the monosome peak fraction of a linear sucrose gradient were cross-linked and put on a similar gradient.

The $S_{20,w}$ values as determined using the McEwen tables for linear sucrose gradients (21) resulted in the same $S_{20,w}$ values for the cross-linked monosomes.

No dimer nucleosomes showed up and the gel pattern of histones extracted from the second gradient is perfectly identical to those

Figure 2. (A) The monosome fraction of a micrococcal nuclease digestion of nuclei, was purified on a 9 ml linear 6%-22% sucrose gradient, layered on a 1 ml 30% sucrose cushion, in the SW41 Beckman rotor, using the band-forming cap equipment. The running conditions were: 32.00 rpm, 5°C, 15 hours. The shaded monosome area was recovered, concentrated, and dialysed against 70 mM phosphate buffer pH 8. After the cross-linking reaction was performed, the cross-linked monosomes were re-run as illustrated in B. (B) The gradient used in this case was a 9 ml linear 8-30% sucrose gradient, layered on a 35% sucrose cushion. All running conditions were the same as in A, except a running time of 17 hours. The O.D.₂₆₀ profile of the punctured tubes was recorded by pumping the gradients through a flow-cell. Our puncturing equipment included a device, giving a sharp mark at the bottom and top of the punctured tubes.

previously described (Fig.2).

The mechanism of dimer formation with MAI. The formation of cross-links with MAI is not unexpected, although it is a monofunctional imidoester. The possibility of such an occurrence has been suggested by Kent and Browne (22) and in a very recent paper on chromatin amidination (6) the occasional occurrence of dimeric material was mentioned. The reaction mechanism of amidination itself clearly suggests such a possibility (5). In an initial step the protonated form of the imidoester attacks an unprotonated amine function (lysine NH_2 in a protein case) to form an intermediate tetrahedral complex. At high pH values ($\text{pH} > \text{pH}_{\text{maximum}}$ which is observed in a pH vs. rate constant curve), this addition step is rate determining and an amidine is formed irreversibly in a fast step from this intermediate complex.

At pH values $< \text{pH}_{\text{maximum}}$ however, the rate determining step is the decomposition of this tetrahedral complex. This complex breaks down along different pathways. These are decomposition and regeneration of

the original reagents, irreversible formation of a monosubstituted amidine or reaction with a nearby amino group which generates a disubstituted amidine. It is this last reaction which is responsible for the observed cross-linkings. All the detailed pathways have been summarized in a recently published scheme (23). One further observation of crucial importance to understand the imidoester behaviour is that the rates of hydrolysis of the reagents are much higher at the low physiological pH values (pH 7.0 - pH 8.0) than at pH 9 or 10 (22).

In view of all this, one can understand the nucleosome cross-linking patterns as a function of pH, with DSI and MAI as shown in Fig.3. DSI, having two imidoester functions available, reacts to such an extent at pH 10, that no mono-, di- or trimeric material remains and only a high molecular weight band is observed. The very high reaction extent reflects the much lower hydrolytic decomposition of the reagent at this pH.

MAI, which only has disubstituted amidine formation as a pathway

Figure 3. Cross-linking patterns of nucleosomal histones at different pH values with MAI (A8, A9, A10 : pH 8, 9, 10 respectively) and DSI (S8, S9, S10 idem). (pH 8.0 reactions were performed in phosphate buffer ($I=0.2$) pH 9.0 and pH 10.0 in borate buffers ($I=0.2$)). All reactions at same nucleosome concentrations ($O.D._{260} = 3.5$) and a 3 mg/ml imidoester concentration. The densitometric scans of the MAI patterns show decreasing amounts of dimeric and higher oligomeric material with increasing pH. The DSI patterns show the disappearance of monomeric histones at pH 9.0 and higher molecular weight material clearly builds up at higher pH values, just beneath the spacer gel-running gel boundary (see photograph).

towards cross-linking, shows, despite the lower hydrolysis rate at this high pH value, a much lower extent of cross-linking because disubstituted amidine formation occurs to only a low extent at high pH. Therefore lower pH values are required to produce oligomers in higher yields.

Extent and identification of dimers formed with MAI. The amount of cross-linked material observed with MAI is quite high. One can best appreciate this from a comparison with an experiment in which lysozyme was cross-linked to itself at different concentrations ranging from 250 $\mu\text{g/ml}$ to 10 mg/ml .

Only at 10 mg/ml and with a high overloading of the material on the gel, a clearly visible lysozyme dimer is observed. In all our nucleosomal experiments cross-linking was nucleosome concentration independent and experiments were performed with histone concentrations in the range of 100-250 $\mu\text{g/ml}$.

This implies that the histone amino groups must be in very close contact in order to generate such an amount of clearly visible oligomers.

The pattern itself already suggests an identity between the dimers formed with DAI, DSI and MAI.

Bands I, II, III generated with DSI have previously been identified as I: H3-H3; II: H2A-H2B; III: (H3-H4) + (H2H-H4) + (H2B-H4)(27). (For this identification, use was made of the analogous pattern obtained with cleavable $-(\text{CH}_2)\text{-S-S-}(\text{CH}_2)_2\text{-}$ linkers).

We used several approaches to identify the products with MAI.

A) - band I' matches perfectly in between the two bands obtained by oxidation and subsequent CYS-CYS dimerisation of pure histone H3 (Fig.4 slot g).

- the position of band II' coincides with the position of the relatively broad band of H2A-H2B dimer cross-linked with DSI (Fig.4 slots e, f)

- band III' coincides with the strongest intensity band arising from H3-H4 tetramer cross-linking (Fig.4 slots b,c) and is very likely H3-H4.

A weaker intensity band formed with this tetramer is due to H4-H4 cross-linking. This dimer was never observed with nucleosomes.

Finally the weakest dimer, only observable at relatively high loading (visible in Fig.4 slot c but not in Fig.4 slot b) matches with band I and therefore presumably arises from H3-H3 linking.

B) Ammonolysis of bands isolated from gels: All four bands, including

Figure 4. SDS polyacrylamide patterns of DSI cross-linking of various histone populations. (a) Control total calf thymus histone showing monomeric band positions. The 2 upper bands are histone H1 fractions. (b) (H3-H4) tetramer (c) idem as (b) but at a higher gel load (d) cross-linked nucleosome (e) H2A-H2B (f) idem at low gel load and after longer reaction time (g) control reduced and oxidized H3 showing the two bands formed by the low amount of the disulfide bridged dimers.

Ia, were cut from a preparative gel and rerun after ammonolysis which is known to cleave amidine linkages. Relatively high ammonolysis times were used (50 hours). This is due to the high degree of insolubility of highly basic proteins in the cleavage medium.

As can be appreciated from Fig.5

- Band Ia generates only H3 - So it must be a second type of H3-H3 dimer.
- Band I' also generates only H3 and thus behaves like the single H3-H3 band which occurs in DSI cross-linking.
- Band II' gives very intense H2A and H2B monomers.
- Band III' has not yet been extracted as a single pure band.

There is always a large amount of band II' coextracted. Short ammonolysis times (8-12 hours) generate a monomer pattern as in figure 5: with a relatively strong H4 band and equally intense H2A, H2B, H3 lines. In longer ammonolyses, H2A and H2B, presumably coming from the band II'

Figure 5. Ammonolysis of cross-linked dimers and resulting monomers. (A) 50 hours ammonolysis (1) band II' (2) control whole histone (3) band I (4) band Ia (B) 12 hours ammonolysis of band III'.

contamination, grew more intense. We suggest H2A and H2B are held together in complex II' with on average more cross-linkages than in the other dimers so that longer cleavage periods are required.

However, because of the II' contamination, the result of band III' cleavage still leaves us in doubt as to whether H2A and H2B occur in dimers migrating at this position in the same way as was shown in the case with DSI (H2A-H4; H2B-H4).

C) In order to clarify this point, autoradiography was performed on gels of cross-linked histones of reconstituted nucleosomes. In this procedure (previously used by Martinson and Mc Carthy (16) for identification of photochemically produced cross-links), H2A or H2B lightly labelled with C^{14} -CH₃ groups was recombined with the other three unlabelled purified individual histones. The reconstituted material showed identical trypsin digestion behaviour as native nucleosomes (this is a good criterion for the reconstitution (18)) and identical cross-linking patterns were obtained. These are also indicative of exact reconstruction since, as an example cross-linking in denaturing conditions (6 M urea) or in very high salt (70 mM phosphate - 2 M NaCl, pH 8.0) conditions gave significant differences in the dimer pattern, especially in the relative band intensities.

Figure 6 shows the autoradiographic profile of such a reconstitution with radioactive H2A'. It is clear that besides the very intense darkening at the position coinciding with band II', a weaker line at position III' can be observed. Reconstituates containing H2B generate a perfectly identical pattern of dimers.

Figure 6. Autoradiography of polyacrylamide gel of cross-linked reconstituted nucleosome containing radioactively labelled H2A.

From these experiments we can conclude that the very short range cross-linker MAI produces the same dimeric products as DAI and DSI plus an additional H3-H3 dimer.

It is clear we cannot exclude, because of the very close molecular weights of H2A and H2B, the existence of H2A and H2B homodimers with these techniques. The only element arguing against these dimers still being the identical kinetics of disappearance of these monomers during the cross-linking reaction (2).

Generation of band Ia with DSI. It obviously is not clear why DSI only generates band Ia to a very low extent. Bifunctional cross-linking with one of its imidoester groups followed by hydrolysis of the second should generate this band in the same way as MAI can.

Careful inspection of gels from material cross-linked with DSI at pH 8.0 ($I = 0.2$) shows the existence of a very weak Ia line (Fig.4). This line can be brought to a higher intensity when reaction is performed in very low ionic strength buffer: triethanolamine $5 \cdot 10^{-4} M$, pH 8.0. A plausible explanation can therefore be offered: after bifunctional reaction of one imidoester function of DSI the second imidoester group is brought in close proximity of another histone's lysine and the dimer disappears and is transformed into higher oligomeric material. On lowering the ionic strength however, the reactivity of lysine- NH_2 groups decreases (it can be involved in a stronger salt linkage with a DNA-phosphate), as was evidenced in lower reactivity indices in competitive labelling experiments by Malchy (24) and hydrolysis can overrun amidination, resulting finally in band Ia generation, reflecting a second mode of H3-H3 cross-linking.

Location of cross-linked residues on trypsin cleavable regions:

In a first attempt to map the positions of cross-links on the individual histones, combined cross-linking and trypsin digestion experiments were performed. It is well established that limit digestion of nucleosomes with trypsin results in a particle in which the 20-30 N-terminal amino

Figure 7. Combined cross-linking and trypsin digestion experiments. (A) 1) Slot showing positions of indicated individual histones and H2A-H2B dimer band allowing comparison with digestion bands. 2) Trypsin digestion and subsequent cross-linking. 3) Cross-linking and subsequent trypsin digestion. (B) 1) Trypsin digestion and subsequent cross-linking (idem as A2) 2) Control slot of trypsin digested non cross-linked nucleosomes.

acids of the histones are cleaved (25).

Fig.7 shows the histone gel pattern resulting from DSI cross-linking and limit digestion as well as the pattern from reversed order treatments.

The limit digest pattern of DSI or MAI cross-linked material is identical to the one resulting from limit digestion of non cross-linked material. No bands, resulting from cross-linking of non digestible histone cores, between limit digest fragments and histone dimer positions, are observed.

The greatest amount (if not all) cross-linking thus occurs on these, operationally defined, trypsin cleavable regions or histone "tails" as they have been referred to earlier.

The reversed order treatment using identical reaction times produces a series of bands, presumably originating from cross-linking of lysines capable of reaction after the trypsin treatment. This oligomeric series of cross-linked digestion fragments was not observed with MAI, plausibly reflecting greater distances between the lysines responsible for this phenomenon.

CONCLUSIONS

There is a vast literature now on chemical cross-linking of histones, most of which is difficult to interpret in view of reagent span lengths. Therefore, monofunctional imidoesters, like MAI, can be very valuable tools for probing very close contacts, when used in the physiological pH range, where disubstituted amidine formation becomes significant.

More in particular, this is true for the chromatin case:

- it was recently demonstrated (6) that even extensive amidination did not alter to any significant extent chemical or physical properties of chromatin (only some properties correlated with the presence of H1 were slightly affected).

- presumably as the result of the fortunately very close contacts between lysine-rich regions in the nucleosome, good yields of cross-linked dimers can be obtained with MAI.

That the cross-linking by MAI is also reversible is a further great advantage. We will try to exploit this further in an attempt to locate these contacts accurately.

Recent results, from a multiplicity of techniques, have led different groups (26) (27) (28) to a disc shaped nucleosome core model, of 110 Å diameter and 57 Å thickness, divided into two symmetrically located half-nucleosomes along the 57 Å axis.

From the 140 b.p. length of core associated DNA, the maximum DNA superhelix radius of ± 45 Å for this core particle and the 80 b.p. separation between highly DNase I sensitive sites the Cambridge group (26) concluded to $1\frac{3}{4}$ superhelical turn of DNA for a core particle or $2\frac{1}{2}$ turns for a 200 b.p. fragment. This bending, putting a high strain on the DNA and the very small separation between two highly negatively charged DNA backbones resulting from this model, demands for a strong stabilizing force between adjacent turns. As a most obvious source of stabilization highly positively charged histone side chains were proposed and these have to occur with a very high local concentration. It is thus plausible that the high yield of very short distance cross-links between trypsin cleavable regions can reflect exactly such an arrangement. The cross-linkings thus appear to refer to histone proximity in the outer DNA-rich parts of the nucleosome.

Especially the observation of an H3 homodimer suggests a very close contact between half-nucleosomes at least at this site.

We are aware that the results were discussed from a static point of

view. It is clear this type of approach cannot escape the criticism that the very short distance cross-links may originate from contacts between dynamically interacting regions having on average greater separations.

This however does not seem very likely to us. Partially proteolysed nucleosomes which generate identical X ray patterns, have identical s values and similar nuclease digestion patterns as intact particles (26). So it is very likely that these lysine-rich fragments are strongly kept in place, despite the fact that some covalent bonds are broken.

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