CLEAVABLE CROSS-LINKS IN THE ANALYSIS OF HISTONE–HISTONE ASSOCIATIONS

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1. Introduction

The cross-linking reagent dimethyl suberimidate has been used to demonstrate a histone octamer in chromatin and free in solution [1]. The histone octamer was found to be associated in chromatin with 200 base pairs of DNA, in agreement with the model for chromatin structure proposed earlier [2].

Further analysis of histone–histone associations by cross-linking required determination of the histone compositions of various cross-linked products. The simplest and most direct way of doing this is by forming cross-links that contain a labile linkage. Here we describe cross-linking of histones in chromatin and in solution through disulphide-containing cross-links that may be cleaved with thiol reagents, and which differ from those formed with dimethyl suberimidate [1] only in replacement of the grouping $-CH_2-CH_2-$ by $-S-S-$. Cross-linking is achieved by monofunctional reaction of histone amino groups with methyl 3-mercaptopropionimidate, followed by oxidation of the newly introduced thiol groups to form disulphide-containing histone–histone cross-links. The cross-linked products can then be analysed for composition by two-dimensional, 'diagonal' [3], SDS-polyacrylamide gel electrophoresis, in which the cross-links are cleaved between the first and second dimension steps. This procedure avoids any misinterpretations that could arise from identification of cross-linked species on the basis of electrophoretic mobility.

In particular we report here that: (1) the dimers formed on cross-linking the four main types of histone in chromatin have the compositions $(F_3)_2$, F2A2·F2B, F2A1·F3, F2A1·FA2, and F2A1·F2B; (2) the octamer that exists free of DNA at high ionic strength contains roughly equal amounts of all four histones, and dissociates on dilution to give a hexamer rich in F2A1 and F3 and a dimer of F2A2 and F2B; (3) the fifth histone, F1, is cross-linked extensively to itself in chromatin. While this work was in progress, reports appeared from other laboratories on the use of disulphide-containing cross-links in studies of ribosome structure [4,5] and of erythrocyte membranes [6].

2. Materials and methods

Native chromatin was prepared from rat liver nuclei as described earlier [7]. The compositions of buffers used for cross-linking at pH 8 (I=0.2), pH 9 (I=0.1), and pH 9 (I=2.0), and the procedures for cross-linking with dimethyl suberimidate, and for one dimensional SDS-gel electrophoresis, were as described [1]; samples containing disulphide cross-links were dissolved in SDS-gel sample buffer from which 2-mercaptoethanol had been omitted.

2.1. *Disulphide cross-linking procedure*

Disulphide-containing cross-links were introduced as follows: to a solution of chromatin in buffer (0.1

mg/ml unless otherwise indicated) was added dithiothreitol in buffer to a final concentration of 10 mM, followed by 0.1 vol of a solution of methyl 3-mercaptopropionimidate (prepared as described [8], m.p. 78°C) freshly dissolved at 11 mg/ml in buffer at 0°C to give a final imidoester concentration of 1 mg/ml; the reaction mixture was kept at 23°C for the time indicated. Hydrogen peroxide solution (30% w/v) was then added to a final concentration of 20 mM and the reaction left for 10 min at 23°C; at 1 min intervals during this period 20 μl samples of the reaction mixture were tested with 1 ml of 5,5′-dithiobis- (2,4-dinitrobenzoic acid) (0.2 mg/ml in 0.1 M Tris–chloride pH 8) to follow the loss of thiol groups, which was complete after 3–4 min. The solution was then incubated at 23°C for 5 min with 2 μl of catalase solution (BDH Ltd., diluted 10-fold with water), transferred to ice, mixed with 0.2 vol of 3M sodium acetate-acetic acid buffer, pH 5, and finally dialysed against 0.2 mM phenylmethylsulphonyl fluoride – 0.1 mM NaEDTA pH 7, and freeze-dried.

The following modifications of the procedure were investigated but had no effect on the band pattern seen in SDS-polyacrylamide gels: (a) omission of dithiothreitol during reaction with the imidoester; (b) dialysis to remove dithiothreitol and excess methyl 3-mercaptopropionimidate before oxidation with hydrogen peroxide; this caused slight precipitation and was therefore avoided; (c) alkylation with iodoacetamide of any thiol groups remaining after oxidation: iodoacetamide was added to a final concentration of 20 mM after the catalase step, and the reaction mixture was kept in the dark at 37°C for 30 min.

When the peroxide oxidation step was omitted, and dithiothreitol was also omitted from the first stage in the procedure, quite extensive cross-linking still occurred, as judged from bands up to hexamer seen in SDS-polyacrylamide gels. This is likely to be due to spontaneous formation of disulphide cross-links under the conditions of reaction with the imidoester at pH 9.

2.2. *Two-dimensional 'diagonal' SDS-polyacrylamide gel electrophoresis*

Two systems were used: (A) dimensions 1 and 2 both SDS-18% polyacrylamide slab gels; (B) dimension 1, SDS-5% polyacrylamide tube gel and dimension 2, SDS-18% polyacrylamide slab gel. System A gives a true diagonal and system B a 'pseudo-diagonal' for samples run in the two dimensions without modification between the two steps.

System A was used for analysis of cross-linked dimers. The slab for the first dimension was 0.15 cm thick and 30 cm long, prepared as described [1]. The slab for the second dimension was 0.4 cm thick and consisted (from bottom to top) of separating gel (24 cm), stacking gel (1.5 cm), both as described [1], and 2 cm of 1% (w/v) agarose [9] containing 0.12 M Tris–Cl pH 6.8, 0.1% SDS (the concentrations present in the stacking gel) and 1.4 M 2-mercaptoethanol. Electrophoresis in the first dimension was carried out as described [1] on 280 μg of protein containing labile cross-links, with the use of a sample well about 2.5 cm wide. The dimer region, which was well-resolved from monomers and trimers, and constant in position in different runs, was cut from the unstained gel, soaked for 50 min in 0.12 M Tris-Cl pH 6.8, 0.1% SDS, 1.4 M 2-mercaptoethanol, then placed on the second dimension slab for electrophoresis at right angles to the first dimension, and set in 1% (w/v) agarose containing 0.12 M Tris–Cl pH 6.8, 0.1% SDS. Electrophoresis was carried out as described [1] at 35 mA for 24 h.

System B was used for analysis of the complete set of trimers, tetramers, and higher multimers. Electrophoresis in the first dimension was carried out in a tube gel 9 cm × 0.4 cm (i.d.) in phosphate buffer [10]. The gel was then soaked and run in the second dimension as described for system A, except that the separating gel was 12 cm long and electrophoresis was carried out at 35 mA for about 16 h.

For best resolution in the second dimension it was essential to make up the agarose in stacking gel buffer, and also to include the layer of stacking gel between the agarose and the separating gel, as described above. However, identical two-dimensional gel patterns could be obtained by omitting agarose and instead placing the mercaptoethanol-treated first dimension gel directly on to the stacking gel layer, and embedding it in more stacking gel, but this procedure suffered from the disadvantage that the thiol greatly inhibited polymerisation of the final stacking gel.

3. Results and discussion

3.1. *Comparison of chromatin cross-linked through disulphide linkages and chromatin cross-linked with dimethyl suberimidate*

In the cross-linking procedure described here, thiol groups that are not converted by oxidation with hydrogen peroxide into disulphide bonds could catalyse disulphide interchange. In chromatin such interchange would be unlikely to generate artifactual cross-linked products since the arrangement of histones is probably fixed; artefacts could however arise from interchange during unfolding of the crosslinked proteins for analysis in SDS-gels.

Two lines of evidence suggest that such interchange either does not occur, or if it does occur then it does not generate new histone–histone associations. The first is that the same cross-linked species are formed in chromatin (or chromatin monomer) when disulphide-containing cross-links are introduced by the two-step procedure described above as after direct bifunctional attack by dimethyl suberimidate (fig. 1a). This is borne out by more detailed analysis of the dimers (fig.1b), although the bands for the disulphide-containing cross-linked products are somewhat diffuse. (The oxidised form of the imidoester, dimethyl bisdithiopropionimidate [9] was not used since it proved to be unstable at pH 9, although relatively stable at pH 8.)

The second piece of evidence against artefacts of disulphide interchange is that alkylation to block any thiol groups remaining after the two-step cross-linking procedure caused no change in the band pattern observed in gels. It therefore seems that no free thiol groups survived oxidation (borne out by lack of any reaction with 5,5′-dithiobis (2,4-dinitrobenzoic acid) at pH 8), or alternatively, but less likely, that any free thiol groups led only to intramolecular disulphide interchange. The absence of free thiol groups after oxidation is probably a consequence of performing the hydrogen peroxide treatment without removing dithiothreitol or excess methyl 3-mercaptopropionimidate. Under these conditions any thiol groups that are not converted into histone-histone disulphide cross-links are instead converted into mixed disulphides with excess reagent.

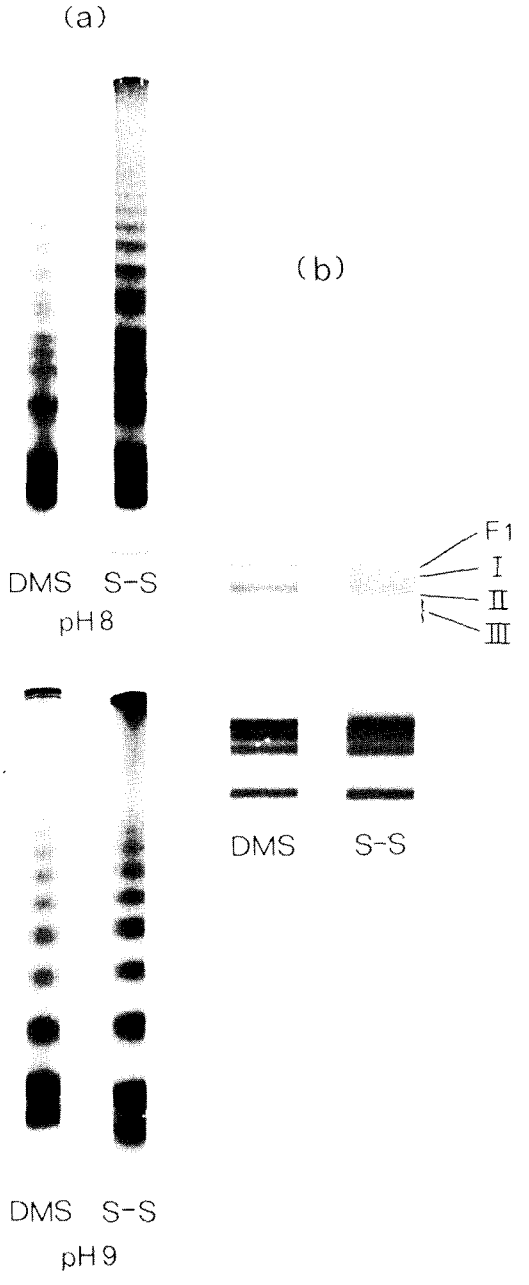

Fig.1. Comparison of chromatin cross-linked through disulphide linkages (S–S) and chromatin cross-linked with dimethyl suberimidate (DMS). (a) Products of cross-linking for 3 h at pH 8, I=0.2, and pH 9, I=0.1, analysed in SDS-5% polyacrylamide tube gels; (b) cross-linked dimers formed after 3 h reaction at pH 8, I=0.2, analysed in an SDS-18% polyacrylamide slab gel.

3.2. *Identification of cross-linked dimers*

The histone dimers in chromatin cross-linked through disulphide linkages were analysed by 'diagonal' SDS-gel electrophoresis in system A (see Materials and methods), and gave the two-dimensional fingerprint shown in fig.2. Four lanes of different monomer composition, I′, II′, IIIa′, IIIb′, may be distinguished (fig.2). These four sets of monomers must have arisen from the three bands, I, II, and III in the dimer region of the gel shown in fig.1b (the slowest moving band in this region is due to Fl monomer). Monomer set I′ contains only F3 and hence I corresponds to an $(F3)_2$ dimer; II′ contains F2A2 and F2B, hence II most probably represents the heterodimer [1] F2A2·F2B; IIIa′ contains F2A1 and F3 and must be due to an F2A1·F3 dimer; IIIb′ contains F2A1, F2A2 and F2B and must arise from a mixture of dimers containing F2A1, namely F2A1·F2A2 and F2A1·F2B.

This assignment of composition to five dimers is in keeping with our previous study [1] of dimers formed by cross-linking with dimethyl suberimidate. In that study, the four dimer bands shown in fig.1b (DMS) were resolved on a longer gel into five bands and assigned on the basis of electrophoretic mobilities to the same five pairs of histones as identified above. Three of the dimers have also been obtained from treatment of sheared chromatin with other reagents: F2A1·F3 from treatment with carbodi-imide [11]; F2A2·F2B from formaldehyde [12]; and F2A1·F2B from formaldehyde [12] and tetranitromethane [13].

If the repeating unit of chromatin structure contains two each of the histones F3, F2B, F2A2 and F2A1 as proposed [2], and if the two F3 molecules, the two F2B molecules etc. are symmetrically placed within the unit, then formation of the cross-linked dimers $(F3)_2$ and F3·F2A1 indicates the existence of an $(F3)_2(F2A1)_2$ tetramer [14] in the repeating unit [1,2]. Cross-linking between F2A1 molecules is evidently not favoured (it is relatively infrequent even in the free tetramer in solution [14]) and F2A1 is instead cross-linked to the F2A2, F2B pair in the repeating unit, as well as to F3.

3.3. *The composition of the histone octamer that exists free in solution at high ionic strength*

Histones dissociated from chromation at high ionic strength (I=2) and pH 9 exist in solution as an octamer and free F1, as shown by cross-linking with dimethyl suberimidate [1]. The relative rates of cross-linking of the four histones F3, F2B, F2A2 and

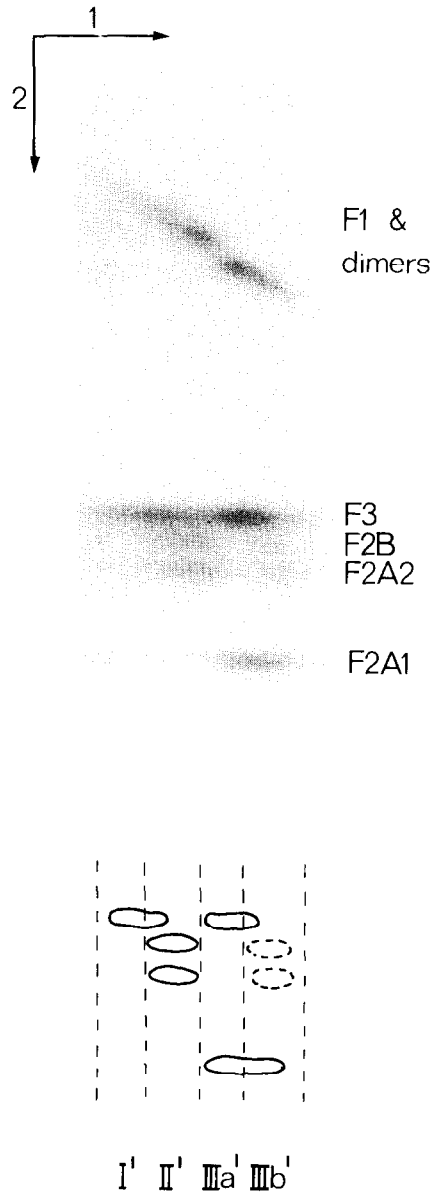

Fig.2. Two-dimensional 'diagonal' gel-electrophoresis (system A) of the histone dimers formed through disulphide-containing cross-links at pH 9, I = 0.1, after 3 h reaction. Traces of F1 and residual dimer lie on the diagonal.

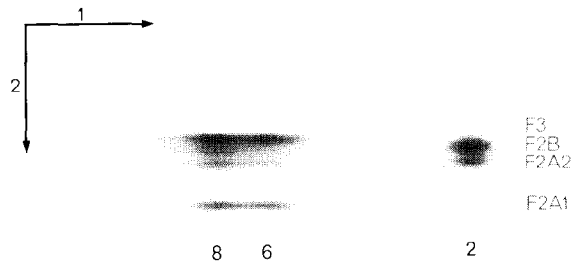

Fig.3. Two-dimensional 'diagonal' gel-electrophoresis (system B) of chromatin cross-linked at pH 9, I = 2.0, for 30 min through disulphide-containing cross-links. The monomer region of the second dimension gel is shown; the band pattern in the first dimension was essentially as shown in fig.4, second gel lane.

F2A1 in this octamer, and the compositions of the dimers formed as intermediates in the reaction, were the same as for histones in chromatin [1], suggesting that the octamer in free solution at high ionic strength is identical in composition with the octamer in chromatin. When the chromatin concentration was lowered there was some dissociation of the octamer into hexamer and dimer [1] (see also fig.4).

The composition of the histone octamer, and of the hexamer and dimer derived from it, were determined by forming disulphide-containing cross-links and then analysing the products by 'diagonal' SDS-gel electrophoresis (fig.3). Despite some streaking in the first dimension, it is clear that the octamer contains roughly equal amounts of F3, F2B, F2A2 and F2A1. The hexamer contains all four histones but less of F2A2 and F2B than of F3 and F2A1, whereas the dimer contains only F2A2 and F2B. The dissociation of the octamer into a hexamer containing all four histones and a dimer containing only F2A2 and F2B is evidence for an unique octamer. (If a mixture of octamers, for example octamers containing only F3, F2A1 and octamers containing only F2A2, F2B, were to dissociate to hexamers, then dimers containing all four histones would be observed.)

The pattern of dissociation also suggests that the arrangement of histones within the octamer is based on two F2A2·F2B dimers in association with an $(F3)_2(F2A1)_2$ tetramer. (Further dissociation of the octamer, on lowering of the chromatin concentration to 0.05 mg/ml or less, gives a tetramer (fig.4); the composition of this tetramer has not been established.)

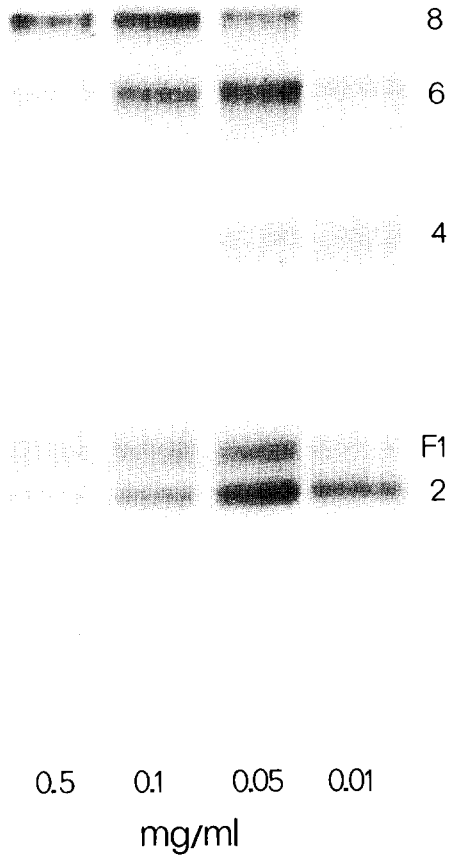

Fig.4. SDS-18% polyacrylamide slab gel electrophoresis of chromatin cross-linked at various concentrations with dimethyl suberimidate at pH 9, I = 2.0, for 1 h.

3.4. *Self-association of F1 in chromatin*

When disulphide-containing cross-links are introduced into the histones in chromatin to the extent shown in fig.1a, the result of two-dimensional gel analysis is as shown in fig.5. A greater proportion of F1 than of the other histones is converted to a high mol. wt product that is excluded from the first dimension gel. (Similar observations on cross-linking of F1 have recently been reported by other workers [11,15]. This could arise from contact between F1 molecules

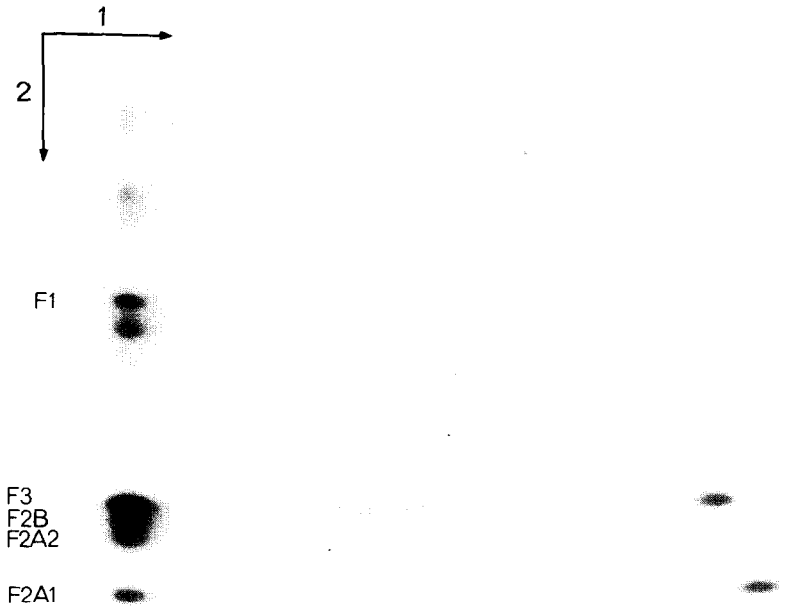

Fig.5. Two-dimensional 'diagonal' gel electrophoresis (system B) of the histones in chromatin cross-linked through disulphides at pH 9, I = 0.1, for 30 min. The band pattern in the first dimension was identical with that shown in fig.1a.

on adjacent chromatin subunits. The elongated shape of F1 suggested by hydrodynamic studies [14,16] is also consistent with a picture in which a chain of F1 molecules, one on the outside [2] of each repeating unit, runs along the chromatin fibre.

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References

[1] Thomas, J. O. and Kornberg, R. D. (1975) Proc. Natl. Acad. Sci. USA 72, 2626–2630.
[2] Kornberg, R. D. (1974) Science 184, 868–871.
[3] Brown, J. R. and Hartley, B. S. (1966) Biochem. J. 101, 214–228.
[4] Traut, R. R., Bollen, A., Sun, T.-T., Hershey, J. W. B., Sundberg, J. and Pierce, L. R. (1973) Biochemistry 12, 3266–3273.
[5] Sommer, A. and Traut, R. R. (1974) Proc. Natl. Acad. Sci. USA 71, 3946–3950.
[6] Wang, K. and Richards, F. M. (1974) J. Biol. Chem. 249, 8005–8018.
[7] Noll, M., Thomas, J. O. and Kornberg, R. D. (1975) Science 187, 1203–1206.
[8] Perham, R. N. and Thomas, J. O. (1971) J. Mol. Biol. 62, 415–418.
[9] Wang, K. and Richards, F. M. (1974) Israel J. Chem. 12, 375–389.
[10] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[11] Bonner, W. M. and Pollard, H. B. (1975) Biochem. Biophys. Res. Comm. 64, 282–288.
[12] Van Lente, F., Jackson, J. F. and Weintraub, H. (1975) Cell, 5, 45–50.
[13] Martinson, H. G. and McCarthy, B. J. (1975) Biochemistry, 14, 1073–1078.
[14] Kornberg, R. D. and Thomas, J. O. (1974) Science 184, 865–868.
[15] Chalkley, R. and Hunter, C. (1975) Proc. Natl. Acad. Sci. USA 72, 1304–1308.
[16] Edwards, P. A. and Shooter, K. V. (1969) Biochem. J. 114, 227–235.