COVALENT CROSS-LINKING OF HISTONES IN CHROMATIN

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Received 1 November 1974

1. Introduction

There is currently much interest in the possibility that chromatin (DNH) may consist of a repeating nucleoprotein subunit. The idea is supported by the in vivo degradation of DNA in chromatin to a series of discrete size classes [1] and by recent studies on isolated histones which report specific interactions between fractions [2–4]. Furthermore, studies in this laboratory have suggested that in the intact chromatin the histones are arranged in a regular, repeating manner along the DNA [5–8]. To obtain more direct information about the spatial relationships between the histones in chromatin, we have covalently cross-linked the proteins using formaldehyde [9]. To aid the interpretation of the results we have cross-linked not only native DNH but also DNH which is specifically depleted of f1 and which is still in the supercoil conformation [10,11] or of f1, f2a2 and f2b where the rigid supercoil properties have been lost [12]. The nucleoproteins have also been treated with formaldehyde in different NaCl concentrations to study the effects of conformational changes and differential dissociation of histones on the reaction. The products have been examined by polyacrylamide-SDS gel electrophoresis.

The results show that when dissociated from DNA in high salt f2a1 and f3 undergo extensive polymerisation, the polymerising unit being a dimer of f2a1 and f3. When bound to DNA f2a1 and f3 can interact extensively to form high mol. wt polymers (apparent mol. wt 170 000 and above) with the dimer again being the basic polymerising unit. Similar behaviour is found for f2a2 and f2b although the two polymerising systems do not appear to interact with each other. Free f1 does not appear to cross link to itself intermolecularly or to any other histone.

2. Methods

DNH was prepared [13] and characterised [11] as described previously. DNH specifically and completely depleted of f1 (0.7 DNH, gel 3.1) or f1, f2a2 and f2b (1.2DNH, gels 1.1 and 2.1) was prepared by dialysis for 3 hr at 4°C against 0.7 M or 1.2 M NaCl, 10 mM sodium phosphate, pH 6.2, respectively [12], followed by gel filtration on Sepharose 4B at 4°C. Protein and DNA concentrations were measured as described previously [11]. Stock HCHO was adjusted to pH 7.0 with NaOH and added to nucleohistone (100–200 μg DNA/ml) to a final concentration of 1%. The solution was incubated for 18 hr at 20°C. NaCl concentrations were adjusted prior to addition of HCHO by dialysis for 3 hr at 4°C. In the following, low salt refers to o.7 mM sodium phosphate, pH 6.8. After reaction the mixture was dialysed into 1% SDS, 4M urea, 0.01 M glycine, pH 10.0, and analysed directly on SDS-polyacrylamide gels at pH 10.0 [14,15]. After staining gels were scanned on a microdensitometer and the traces analysed on a curve analyser [7]. Polyacrylamide-urea gel electrophoresis was performed by the method of Panyim and Chalkley [16].

Accurate mol. wt estimation of cross-linked products is not possible on SDS gels because histones migrate anomalously [14,15] and because covalent cross-linking may itself affect the conformation in SDS. We have estimated mol. wt (M_{app}) taking f2a1 and f3 as standards since these two fractions are common to all

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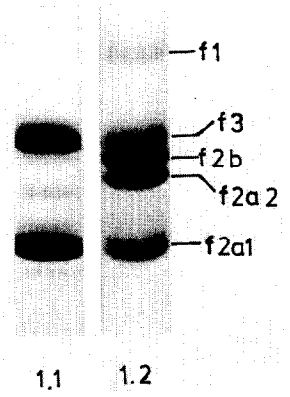

Fig. 1. Polyacrylamide-urea gels of (1.1) histones extracted from 1.2 DNH and (1.2) histones extracted from native DNH.

control gels. The yields of the individual bands and their apparent mol. wt are shown in fig.1.

Total yields in high salt were usually between 70–100%.

3. Results

The sedimentation of HCHO-treated DNH was similar to controls indicating that any cross-linking was restricted to individual DNH molecules. Higher concentrations of HCHO (up to 8%) did not alter the gel pattern showing that in 1% HCHO a reaction limit was reached. Extensive digestion of HCHO-treated material with DNAase I (after removal of excess reagent) before gel analysis did not alter the gel pattern. Covalent linkages between protein and DNA are thus not significant.

a) 1.2 DNH (fig.2)

The only histones bound to DNH in this sample are f3 and f2a1 [gel 1.1]. Approximately 50% of these

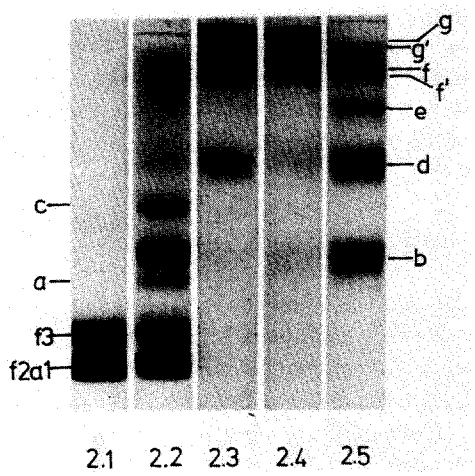

Fig. 2. Polyacrylamide-SDS gels of 1.2 DNH treated with 1% HCHO. (2.1) in low salt (control – no HCHO). (2.2) in low salt. (2.3) in 0.7 M NaCl. (2.4) in 1.2 M NaCl. (2.5) in 2.0 M NaCl.

Table 1

Band Position		*b*	*h*	*d*	*e*	*f*	*g*
M_{app}		29 000	38 000	64 000	119 000	170 000	?
Gel	(2.3)			16		52	+
	(2.4)	4		5		41	+
						(f'–155 000)	(g'–186 000)
	(2.5)	29		27	10	(8)	(4)
	(3.3)					19	+
	(3.4)	32			7	31	+
	(3.5)	50		10	12	27	+
	(4.3)		13			42	+
	(4.4)	32	19		8	26	+
	(4.5)	31	17	14	13	20	

Percentage yields of individual bands relative to total histone on controls taken as 100% (gels 2.1, 3.1, & 4.1). Samples in low salt omitted for clarity. '+' signifies presence of band in *g* position. It is not possible to quantitate bands in this position.

histones reacted with HCHO to form higher mol.wt species in low salt (bands a,b,c,d), the major component b (25% of total protein) having M_{app} = 29 000. Equal amounts of f2a1 and f3 are involved in the reaction since the ratio of the unreacted fractions (27:26) is very close to the ratio in the control (52:48). In 0.7 M NaCl, except for a minor band (d, M_{app} = 64 000) all the protein is polymerised, over half into a species f with M_{app} = 170 000 and the rest into a possibly heterogeneous component g that hardly penetrates the gel. In 1.2 M NaCl band d disappears otherwise the pattern remains the same. In 2.0 M NaCl both histones were completely dissociated from the DNA and a series of oligomeric species was observed (b, d, e, f', g') with M_{app} = 27 000, 64 000, 119 000, 155 000 and 186 000. These closely approximate to a ratio of 1:2:4:5:6.

b) 0.7 DNH (fig. 3)

0.7 DNH, when cross-linked in low salt, gave rise to a series of minor bands with M_{app} in the range 26 000–83 000 and a high mol. wt component (M_{app} = 170 000). These bands, together with the free histones, identified as f2a1 and f3, accounted for about 50% of the total protein. In 0.7 M NaCl all four histones are polymerised to give component f (M_{app} = 170 000), although, as in the low salt case, there is evidence of very high mol. wt material not moving into the gel. In 1.2 M NaCl, where f2a2 and f2b are completely and discretely dissociated, new bands arise (b, M_{app} = 29 000 and e, M_{app} = 119 000) together with bands f, and g. Comparing this result with that in 1.2 M NaCl we conclude that bands b and e arise from cross-linking of dissociated f2b and f2a2. Together, they account for about 75% of these two fractions. In 2.0 M NaCl a series of oligomeric species was again observed, the pattern being qualitatively similar to that observed for 1.2 DNH, but here, the major components are in positions b and f.

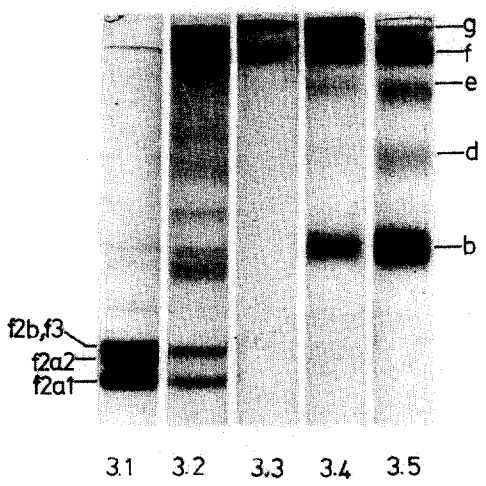

Fig. 3. Polyacrylamide-SDS gels of 0.7 DNH treated with 1% HCHO. (3.1) in low salt (control – no HCHO). (3.2) in low salt. (3.3) in 0.7 M NaCl. (3.4) in 1.2 M NaCl. (3.5) in 2.0 M NaCl.

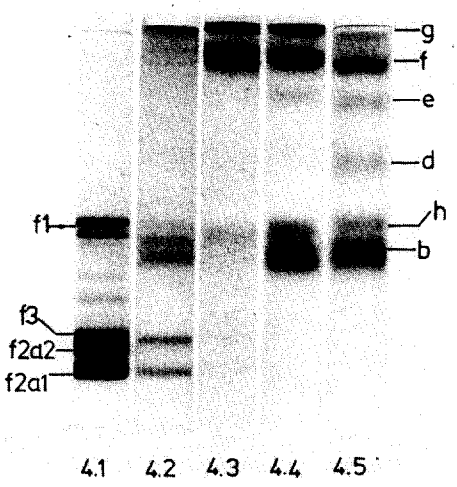

Fig. 4. Polyacrylamide-SDS gels of native DNH treated with 1% HCHO. (4.1) in low salt (control – no HCHO). (4.2) in low salt. (4.3) in 0.7 M NaCl. (4.4) in 1.2 M NaCl. (4.5) in 2.0 M NaCl.

c) Native DNH (fig. 4)

Cross-linking DNH in low salt produced a pattern very similar to that of 0.7 DNH in the same solution, with the exception that band f was absent. About 40% of the material can be accounted for on the gel. In 0.7 M NaCl where f1 is completely dissociated all of the remaining four fractions were cross-linked to give high mol. wt products (bands f and g; cf 0.7 DNH under similar conditions) together with band h. In 1.2 M NaCl and 2.0 M NaCl the patterns are identical to those of 0.7 DNH in the same solutions except that band h is again present, representing just under 20% of

the total protein. From this comparison and its mobility relative to native f1, we conclude that band h represents f1, possibly cross-linked intramolecularly.

4. Discussion

HCHO covalently bonds the histones in DNH to give a discrete series of high mol. wt products, the degree of polymerisation being dependent on ionic strength. Free histones in high salt also show polymerisation behaviour similar to, but not identical with, histones bound to DNA. The simplest case studied is 1.2 DNH which has only f2a1 and f3 bound to DNA. In 2.0 M NaCl where both fractions are completely dissociated, the smallest mol. wt species b may be identified as a dimer of f2a1 and f3, because (1) M_{app} = 29 000, close to the theoretical value of 27 000; (2) the mol. wt of the larger products are integral multiples of this value, suggesting that the dimer is the basic unit of polymerisation and that other combinations of the two histones are absent; (3) f2a1 and f3 reversibly dissociate as an equimolar complex over the range 1.2 to 2.0 M NaCl [12] and the mol. wt of this complex must be at least that of the dimer; (4) when f2a1 and f3 remain uncross-linked (e.g. gel 2.2) they are always in equimolar proportions. Although the smallest mol. wt species is the dimer, higher polymers are also found in significant amounts. When f2a1 and f3 are bound to DNA the degree of cross-linking is low at low ionic strength, with the dimer being the major cross-linked species. However, there are significant amounts of free f2a1 and f3 present which suggest that there is a monomer-dimer equilibrium, which lies heavily in favour of the dimer at high ionic strength, but which is displaced in favour of the monomer in low salt. In the latter case the interactions between f2a1 or f3 and DNH are presumably stronger than the protein-protein interactions. The formation of discrete high mol. wt polymers in 0.7 and 1.2 M NaCl suggests that long-range conformational changes (which result from the flexible, polyelectrolyte behaviour of 1.2 DNH [6]) bring the histone dimers into close association and enable extensive cross-linking to occur. The very high mol. wt polymers observed under these conditions (band g) are not present in significant amounts in free histone. This shows that the histones are capable of much more extensive intermolecular interaction in the bound form compared to the free state.

The additional effect of f2a2 and f2b on this cross-linking pattern is seen by comparing the behaviour of 0.7 DNH with 1.2 DNH. In 0.7 M NaCl where all proteins are bound, the major species have M_{app} = 170 000 and higher as was found for 1.2 DNH in the same salt. The observation that the same polymers exist in the cross-linked supercoiled form (0.7 DNH in 0.7 M NaCl) as in 1.2 DNH in high salt implies that the flexible polyelectrolyte behaviour of the latter in these conditions results in a conformation which approximates to that of the supercoil. In the case of 0.7 DNH in 1.2 M NaCl, where f2a2 and f2b are completely dissociated and f2a1 and f3 are completely bound, a species of M_{app} = 29 000 is prominent (gel 3.4), suggesting that f2a2 and f2b also exist as a dimer when free (mol. wt expected = 28 400) and thus possibly also when bound to DNA. A comparison of gels 2.4 and 2.5 and gels 3.4 and 3.5 shows that the polymerisation of the f2a1/f3 system and the f2a2/f2b system occurs independently both when histones are bound and when they are free, since no new mol. wt species are formed when both are present. The coincidence of the mobilities of bands arising from the two systems is to be expected considering the small difference in the mol. wt of the two dimers which are the basic polymerising units.

The presence of free f1 has no effect on the interactions of the other four histones. It is not cross-linked to these or itself in high salt, although its behaviour in low salt, where it is bound to DNH, is not clear. Intramolecular modification is a possibility in both cases.

Recent studies on the interactions of isolated f2a1 and f3 in solutions of moderate ionic strength (0.1) have revealed either dimer-tetramer equilibrium [2] or a stable tetramer [3]. Similar studies on f2b and f2a2 show a dimer as the major species [4] or a mixture of monomers, dimers and larger polymers [3]. Our studies show that higher polymers of f2a1 and f3 than the tetramer can form in high salt, and that in low salt, the dimer form predominates. This does not support the view that the tetramer has a unique role to play in chromatin structure [17]. Indeed higher polymers of both systems may be more relevant to the structures that histones have in chromatin, since similar polymeric species are observed when histones are

bound to DNA. It is possible that close interaction between dimeric species may be the major factor involved in packing the DNA into the chromatin supercoil.

Acknowledgement

J. E. H. acknowledges financial support from the Medical Research Council.

References

[1] Burgoyne, L. A., Hewish, D. R. and Mobbs, J. (1974) Biochem. J. 143, 67–72.
[2] Roark, D. E., Geoghegan, T. E. and Keller, G. H. (1974) Biochem. Biophys. Res. Comm. 59, 542–547.
[3] Kornberg, R. D. and Thomas, J. O. (1974) Science 184, 865–868.
[4] Kelley, R. I. (1973) Biochem. Biophys. Res. Comm. 54, 1588–1594.
[5] Henson, P. and Walker, I. O. (1970) Eur. J. Biochem. 16, 524–531.
[6] Henson, P. and Walker, I. O. (1971) Eur. J. Biochem. 22, 1–4.
[7] Chatterjee, S. and Walker, I. O. (1973) Eur. J. Biochem. 34, 519–526.
[8] Hyde, J. E. and Walker, I. O. (1974) Nucl. Acids Res. 1, 203–215.
[9] Fraenkel-Conrat, H. and Olcott, H. S. (1948) J. Biol. Chem. 174, 827–843.
[10] Garrett, R. A. (1971) Biochem. 10, 2227–2230.
[11] Henson, P. and Walker, I. O. (1970) Eur. J. Biochem. 14, 345–350.
[12] Burton, D. R., Hyde, J. E. and Walker, I. O. Unpublished observations.
[13] Zubay, G. and Doty, P. (1959) J. Mol. Biol. 1, 1–20.
[14] Panyim, S. and Chalkley, R. (1971) J. Biol. Chem. 246, 7557–7560.
[15] Hayashi, K., Matsutera, E. and Ohba, Y. (1974) Biochim. Biophys. Acta 342, 185–194.
[16] Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337–346.
[17] Kornberg, R. D. (1974) Science 184, 868–871.