A NUCLEOSOME-LIKE PARTICLE CONTAINING AN OCTAMER OF THE ARGININE-RICH HISTONES H3 AND H4

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1. Introduction

Recent work in many laboratories [1–8] has confirmed the central role of the arginine-rich histones H3 and H4 in the nucleosome [9]. H3 and H4 occur in solution as the tetramer $(H3)_2(H4)_2$ [10,11] and the protein core of the nucleosome probably comprises one such tetramer and two molecules each of the lysine-rich histones H2A and H2B (for references see [12] or [13]). It is generally assumed that those properties of chromatin that are regenerated by reconstitution of DNA and histones H3 and H4 alone, such as nuclease sensitivity [1], a low angle X-ray diffraction pattern [6], a beaded appearance in the electron microscope [3–5], and insertion of supercoils into closed circular DNA [3,4,8], are generated by the combination of one tetramer with a nucleosome core length of DNA (about 140 base pairs). However, without better characterisation of reconstituted complexes it is not possible to assess the extent to which a single tetramer is capable of conferring nucleosome-like properties on 140 base pairs of DNA.

We report here the preparation and partial characterisation of nucleosome-like particles containing two tetramers of H3 and H4 and about 140 base pairs of DNA; these are distinct from a complex containing one tetramer and 140 base pairs. The particles have been prepared in two ways by mild procedures involving dissociation of the lysine-rich histones from chromatin or nucleosome core particles with salt, thus ensuring minimal manipulation of, and damage to, H3 and H4.

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2. Experimental

2.1. *Removal of lysine-rich histones from chromatin and nucleosome core particles*

Long native chromatin (10 ml at A_{260} ca. 20) from rat liver (weight average size about 40 nucleosomes [14]) was brought to 1 M final conc. NaCl, and the complex of H3, H4 and DNA was separated from the dissociated lysine-rich histones by gel exclusion chromatography at 4°C on Sepharose 6B-CL (Pharmacia) equilibrated with 1 M NaCl, 10 mM Tris–Cl (pH 7), 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM sodium bisulphite, 0.2 mM Na_2 EDTA, 0.5 mM 2-mercaptoethanol. The histone content of fractions across the peak of 'stripped chromatin', which emerged at the exclusion volume of the column, was analysed by SDS–gel electrophoresis in discontinuous Tris slab gels [15,16] and those fractions containing only H3 and H4 pooled. The H3/H4 chromatin was dialysed at A_{260} ca. 2 against buffers containing 10 mM Tris–Cl (pH 8) and the following concentrations of NaCl, at 22°C, for the times indicated: 0.85 M (3 h), 0.65 M (3 h), 0.5 M (6 h); and finally dialysed overnight at 4°C against 10 mM Tris–Cl (pH 7) which was initially at room temperature. The product was concentrated about 10-fold by dialysis against polyethylene glycol 6000 (50%, w/v) in 40 mM Tris–Cl (pH 7) then dialysed again against 10 mM Tris–Cl (pH 7) at 4°C.

Nucleosome core particles containing 140 base pairs of DNA and no H1 (prepared by digestion of H1-depleted mononucleosomes [17,18]) were depleted of H2A and H2B, and dialysed to low ionic strength in an identical manner. Products were analysed in 5–20% linear sucrose gradients contain-

ing 10 mM Tris–Cl (pH 7) 0.2 mM Na_2EDTA; rotor speeds and times were as indicated in the figure legends. Gradients were fractionated and monitored by pumping from the bottom through an LKB flow cell at 260 nm.

2.2. *Digestion of H3/H4 chromatin with micrococcal nuclease*

H3/H4 chromatin (A_{260} = 15) in 10 mM Tris–Cl (pH 7) was brought to 0.1 M final conc. NaCl and digested at 20°C in the presence of 1 mM $CaCl_2$ with 105 units/ml micrococcal nuclease (P-L Biochemicals); digestion was terminated by addition of 0.1 M Na_2 EDTA (pH 7) to 10 mM and chilling to 0°C. Digested samples were cleared by brief centrifugation and fractionated in sucrose gradients as indicated above for 'stripped cores'.

2.3. *Protein:DNA ratios*

The DNA concentration of H3/H4 chromatin or particles was determined spectrophotometrically at 260 nm, taking A_{260} = 24 for 1 mg/ml of DNA bound to histone [19]. Histone concentration was determined by amino acid analysis of an acid hydrolysate of chromatin as in [20]; the results were completely consistent with the presence of equimolar amounts of H3 and H4 and the absence of other proteins. (The presence of DNA in the hydrolysis sample resulted in a high value for glycine but otherwise did not seem to affect the amino acid analysis.) Authentic nucleosome core particles analysed by this method gave a protein : DNA ratio of 1.10:1 g/g (calculated value = 1.26:1 g/g).

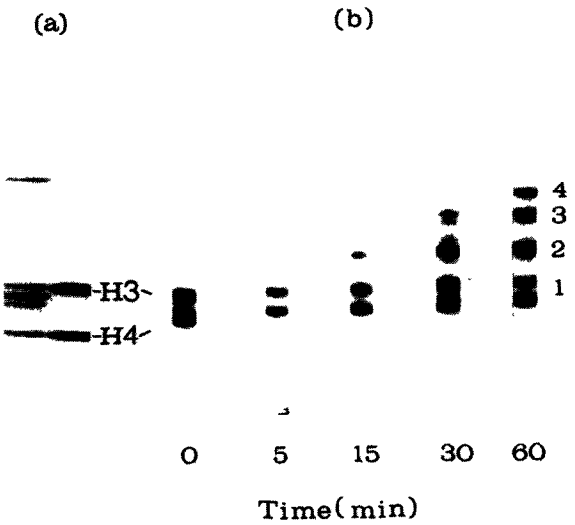

Fig.1. Histone content of H3/H4 chromatin. (a) H3/H4 chromatin run in an SDS–18% polyacrylamide Tris slab gel with total histone as marker; (b) H3/H4 chromatin (A_{260} = 0.3) crosslinked with dimethyl suberimidate (1 mg/ml) in 1.95 M NaCl, 137 mM Na borate (μ=2) (pH 9.0) at 4°C for the times indicated, and run in phosphate–SDS–5% polyacrylamide gels.

2.4. *Miscellaneous methods*

Procedures have been described elsewhere for analysis of DNA in double-strand or single-strand form [21,22], for SDS–polyacrylamide gel analysis of histones and crosslinked histones, and for crosslinking of histones with dimethyl suberimidate [15,16]. Sedimentation coefficients and molecular weights were measured by analytical ultracentrifugation [20,23].

3. Results

3.1. *An H3/H4 particle obtained by digestion of H3/H4 chromatin*

3.1.1. Characterisation and crosslinking of H3/H4 chromatin

The complex of H3, H4 and DNA (H3/H4 chromatin) prepared by removal of the three lysine-rich histones H1, H2A and H2B, and essentially all non-histones (but no H3 or H4) from chromatin by gel-exclusion chromatography in the presence of 1 M NaCl had an H3:H4 ratio identical with that in chromatin, as indicated by densitometry (not shown) of stained slab gels (fig.1a). The protein : DNA weight ratio of the H3/H4 chromatin was 0.42:1, in good agreement with the calculated ratio of 0.43:1 for one $(H3)_2(H4)_2$ tetramer per 200 base pairs of DNA. When H3/H4 chromatin is treated with 2 M NaCl, the histones are released from the DNA as tetramers, as shown by crosslinking (fig.1b).

3.1.2. Digestion of H3/H4 chromatin with micrococcal nuclease

Figure 2 shows the double-strand DNA extracted from H3/H4 chromatin after digestion with micro-

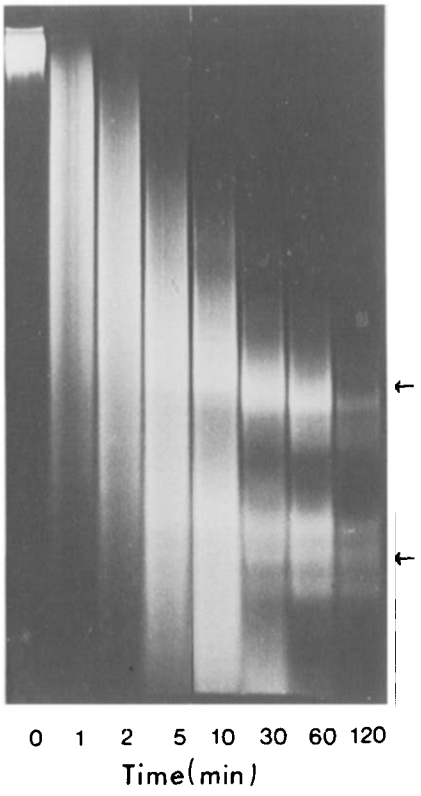

Fig.2. Time course of digestion of H3/H4 chromatin (A_{260} = 6) with micrococcal nuclease (30 U/ml) at 20°C. The extracted DNA was analysed in double-strand form in a 5% polyacrylamide slab gel in Tris–acetate buffer. The upper and lower arrows indicate the approximate positions of 140 and 70 base pair DNA, respectively.

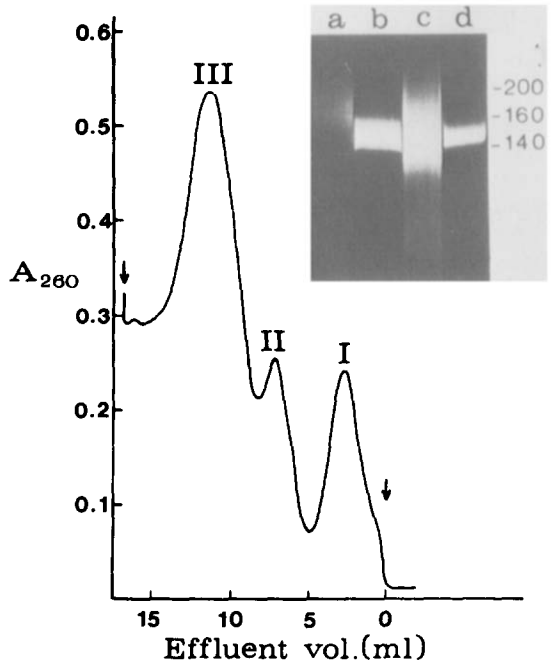

Fig.3. Sucrose gradient profile of H3/H4 chromatin digested for 5 min as in legend to fig.2 (see text). Centrifugation was at 4°C for 51.5 h at 27 000 rev./min in a Beckman SW.27 rotor; the gradients were 16 ml. (Sedimentation was from left to right; arrows indicate top and bottom of gradient). Inset: DNA size of component I from the gradient (gel as in fig.2). (a) Mononucleosomes; (b) and (d) nucleosome core particles; (c) component I.

coccal nuclease for increasing times in the presence of 0.1 M NaCl. (During digestion under these conditions some precipitation occurs (30% of the DNA).) Superimposed on the high background found in the gel at early times of digestion (presumably due to free DNA) are two sets of bands; one around 120–200 base pairs and the other around 70 base pairs. These persist in the later stages of digestion indicating that they arise from metastable intermediates. (When digestion was carried out in 10 mM Tris–Cl alone, only very faint bands at about 140 and 70 base pairs were visible above a very high background of DNA in the gel, and these bands were rapidly degraded further.)

Figure 3 shows the components of a 5 min digest analysed in a sucrose gradient after brief centrifugation to remove the slight turbidity that developed during digestion. The three components were further purified by rerunning in identical gradients. In an isokinetic gradient [24] the sedimentation coefficient of component I was 9.7 S, relative to nucleosome core particles (11.2 S [17]) as marker. Component I contained DNA with a distribution of sizes (120–200 base pairs) centered at 140–150 base pairs (fig.3, inset), and had a protein:DNA ratio of 0.94:1 g/g. (Two tetramers per 120, 140 and 200 base pairs would have ratios of 1.44:1, 1.24:1 and 0.87:1, respectively.) Crosslinking with dimethyl suberimidate at pH 9 showed that component I contained octamers of H3 and H4 (fig.4). Two distinct tetrameric domains in the octamer are suggested by the relatively intense bands due to crosslinked tetramer and octamer, and by a pattern of crosslinked dimers and trimers identical with that obtained for the

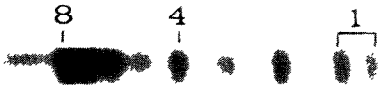

Fig.4. Crosslinking of component I (A_{260} = 1.4) with dimethyl suberimidate (2 mg/ml) at pH 9 (274 mM Na borate, μ=0.1) for 3 h at 21°C. Gel as in legend to fig.1b; migration from left to right.

$(H3)_2(H4)_2$ tetramer crosslinked in solution [10]. Preliminary measurements of the sedimentation coefficient ($s_{20,w}$) of component I in 10 mM Tris–Cl (pH 7.5) in the analytical ultracentrifuge gave a value of 8.7 S; under the same conditions [23] 140 base pair core particles sedimented at 10.4 S. (8.7 S is a weight-average value because of the heterogeneity in DNA size; it may be low because the heterogeneous particles have not been trimmed to remove DNA 'tails'.) The rapidly sedimenting component (fig.3) thus has the properties of a relatively compact particle containing an octamer of the arginine-rich histones and about 120–200 base pairs of DNA, the heterogeneity being a consequence of the method of preparation.

Component II, which has so far been less well characterised, appears to contain a tetramer of H3 and H4, and DNA about half the length of that in component I, visible in double-strand form in fig.2 and giving bands at 60, 70 and 80 bases on a denaturing gel (not shown); component II may therefore be a complex of one tetramer and about 70 base pairs of DNA. Component III from the gradient (fig.3) was essentially free DNA, very heterogeneous and of average size about 70 base pairs as estimated by gel electrophoresis (not shown). Free 70 base pair DNA could arise by breakdown of component II; binding of histones released by such breakdown to fragments of DNA produced during nuclease digestion of H3/H4 chromatin would account for the insoluble material removed before sucrose gradient centrifugation.

3.2. *An H3/H4 particle obtained from 'stripped' 140 base pair nucleosome core particles*

H2A and H2B were removed from 140 base pair nucleosome core particles by gel-filtration in the presence of 1 M NaCl to give 'stripped core particles'. The sucrose density gradient profile of 'stripped cores' after reannealing at 22°C is shown in fig.5. (Reannealing at 4°C gave virtually identical results, as did dialysis to 2 M NaCl to dissociate the histones completely from the DNA before reannealing at 22°C.) Component I′ had the same histone content and pattern of crosslinking (fig.6) as component I obtained by digestion of H3/H4 chromatin (see fig.4); its histone:DNA ratio was 0.94:1 g/g. It differed from component I in having a homogeneous DNA content of about 140 base pairs due to its method of preparation, and a slightly higher sedimentation coefficient ($s_{20,w}$) of 9.1 S. Preliminary measurements of the molecular weight of the particle by sedimentation equilibrium methods, using a partial specific volume ($\bar{v}$) of 0.66 ml/g [25], gave a value of 194 340; the calculated molecular weight for the complex of two tetramers and 140 base pairs of DNA is 200 330. I′ is thus a compact octameric particle.

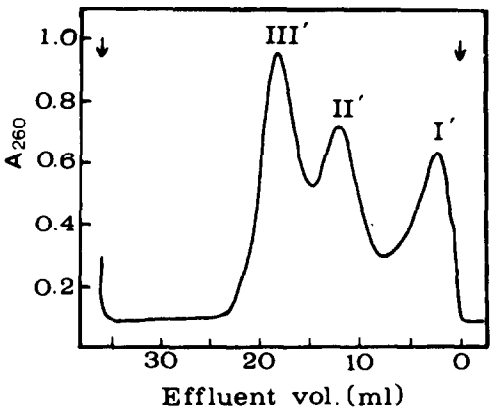

Fig.5. Sucrose gradient profile of 'stripped cores' after reannealing (see text). Centrifugation was at 4°C for 60 h at 27 000 rev./min in a Beckman SW.27 rotor; the gradients were 35 ml. (Sedimentation was from left to right; arrows indicate top and bottom of gradient.)

Fig.6. Crosslinking of components I′ and II′ (A_{260} = 1.8) with dimethyl suberimidate (1 mg/ml) at pH 9 (137 mM borate, μ=0.05) for 1 h at 22°C. Gels as in legend to fig.1b; migration from left to right.

Component II′ from the gradient (fig.5) had a protein : DNA ratio of 0.5:1 g/g, and the histones could be crosslinked to tetramers (fig.6) with dimethyl suberimidate. Preliminary sedimentation measurements gave a sedimentation coefficient ($s_{20,w}$) of 6.0 S and 150 000 mol. wt (using $\bar{v}$ = 0.65 ml/g based on composition of protein and DNA [26]). Component II′ is probably a complex of one tetramer and 140 base pairs of DNA; the calculated molecular weight of such a complex is 146 850. If components I′ and II′ had the same molecular shape, a difference of one tetramer in the molecular weight would not account for the difference between the *S* values, and it is therefore likely that the tetrameric complex is considerably less compact than the octameric particle. Component III′ was essentially free 140 base-pair DNA, probably resulting from migration of a tetramer from one stripped core to another to give the octameric particle. The observed excess of free DNA over octameric particle could arise from instability of the tetrameric or octameric complex; however, we find the octameric complex to be very stable (see section 4).

The organisation of the DNA components in I′ and II′, shown by crosslinking to contain, respectively, an octamer and a tetramer of the arginine-rich histones, was probed by digestion with DNase I. Preliminary results on the pure components (not shown) show bands up to 140 bases on a denaturing gel in the former case and up to 70 bases in the latter, suggesting that 140 base pairs of DNA are strongly bound in component I′ but only 70 in component II′. When an unfractionated mixture of the two components is digested (fig.7) these two sets of bands are superimposed, the net result being strong bands up to 70 bases and a weaker set extending beyond 70 bases up to 140 bases (fig.7, 15 s digest).

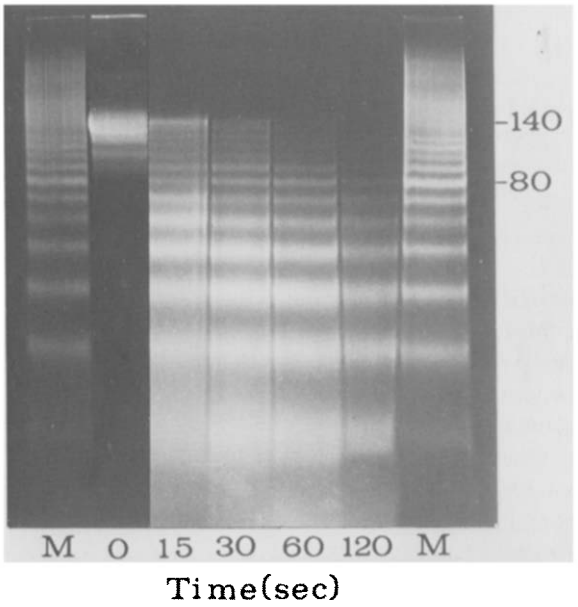

Fig.7. Digestion of an unfractionated mixture of octameric and tetrameric 140 base pair complexes (I′ and II′) (A_{260} = 0.7) with DNase I (30 U/ml) and 10 mM $MgCl_2$ at 20°C. DNA fragments were analysed in a 12% polyacrylamide slab gel containing 7 M urea in Tris–borate buffer; M is a marker DNase I digest of rat liver chromatin. (Minor bands below 140 base pairs in the starting material arise from slight overdigestion with micrococcal nuclease during preparation of core particles.)

4. Discussion

We have shown that when lysine-rich histones are removed from nucleosome core particles under mild conditions in 1 M NaCl, some tetramers of H3 and H4 migrate to other 'stripped cores' leaving free DNA and giving an octameric complex, whose protein content has been demonstrated by crosslinking. Similar octameric particles are formed when chromatin depleted of the lysine-rich histones is digested with micrococcal nuclease, except that the DNA size in this case ranges from 120 to 200 base pairs. There is some evidence (see fig.2) that such particles can be 'trimmed' to a more homogeneous DNA size (c.f., trimming of nucleosomes to core particles [17]), but this has not yet been studied further. On the basis of electrophoretic mobility, Moss et al. inferred [7] that a similar nucleoprotein particle containing two arginine-rich tetramers was formed by digestion of a reconstitute of H3, H4 and long DNA, but this was not characterised further.

The octameric particle resembles the nucleosome core particle in having eight histones, a histone: DNA ratio of 0.94:1 g/g (cf. 1.10 g/g measured for the nucleosome core particle) and sedimentation coefficient ($s_{20,w}$) of 9.1 S (compared with 10.4 S). We find that the octameric particle, a 'pseudonucleo-

some core particle', is stable during routine handling (e.g., dialysis and concentration of sucrose gradient fractions) and for 3 days at 5°C under sedimentation equilibrium conditions in the analytical ultracentrifuge (P. G. Stockley, P. J. G. Butler and J. O. Thomas unpublished observations). A complex containing one tetramer and 140 base pairs of DNA is also formed when lysine-rich histones are removed from core particles, as shown by crosslinking. Its sedimentation coefficient ($s_{20,w}$) of about 6 S shows that it is not compact. Digestion with DNase I has not given any single-strand fragments larger that 70–80 bases in a denaturing gel, suggesting that only half the DNA is protected.

The conclusions drawn here from chemical crosslinking and other methods complement, and are in complete agreement with the recent conclusions of Felsenfeld et al. [27,28] who have shown by the use of sedimentation and nuclease digestion methods that reconstitution of H3 and H4 with 140 base pairs of DNA (in the presence of urea) can give rise to a compact octameric particle and a less compact tetrameric complex. Klevan et al. [26] have studied the tetrameric complex and find the DNA to be only very loosely folded. Our results and those of Felsenfeld et al. would seem to differ from those of Bina-Stein and Simpson [4,5] who identify a compact 9.8 S particle obtained by reconstitution of H3 and H4 with 140 base pairs of DNA as tetrameric.

Since a complex of eight arginine-rich histone molecules (two $(H3)_2(H4)_2$ tetramers) and 140 base pairs of DNA has nucleosome-like properties, as judged by nuclease sensitivity and sedimentation properties, whereas one tetramer does not appear to fold the DNA into stable a compact structure, octameric rather than tetrameric complexes may be responsible for the generation of chromatin-like properties in previous reconstitutions of arginine-rich histones and DNA judged by these criteria. As shown here, such complexes can form even when the initial ratio of arginine-rich histone to DNA is 0.4 g/g. It is equally possible that such particles could be those previously observed by electron microscopy of reconstituted material [3–5] and those responsible for supercoiling of closed circular DNA [3,4,8], especially where the ratio of histone:DNA used was greater than 1:1 by weight. This would imply that in the native nucleosome H2A and H2B (two of each) play an important role in compacting the DNA. In the pseudonucleosome the roles of H2A and H2B are mimicked by H3 and H4.

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