The reconstitution of histone H_3–H_4 tetramer from acid extracted histones in the absence of urea

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Simple mixing of acid purified histones H_3 and H_4 in equimolar quantities at low ionic strength near pH 7 does not yield the tetramer but rather a high M_r aggregate. Dialysis of acid extracted total or core histones into 2 M NaCl 150 mM phosphate (pH 7.4) followed by fractionation of the histone complexes at lower ionic strength (150 mM NaCl) results in an H_3–H_4 tetramer of a structure identical to that derived from salt-extracted histones. Dialysis of acid extracted total or core histones directly into the lower ionic strength buffer with subsequent fractionation, results in H_3–H_4 tetramer of closely similar structure.

Histone *Reconstitution* *H_3–H_4 tetramer* *(Chicken erythrocyte chromatin)*

1. INTRODUCTION

In order to understand histone–histone and histone–DNA interactions and structurally analyse such defined complexes, it is important to develop pathways for the reconstitution of natural histone–histone and histone–DNA complexes.

Methods for the isolation of histones can be divided into two groups; the first results in the isolation of the individual polypeptide chains by employing solvent solubility properties of the histones [1], charge differences of the individual histones in the presence of urea or guanidinium chloride [2] and size-charge and aggregation differences in dilute hydrochloric acid [3]. All these methods have in common that the separation conditions are known to seriously interfere with the higher order structures of proteins. The second group of methods is based on the dissociation of natural histone–DNA complexes at high ionic strength and fractionation of the resulting histone–histone complex utilizing the ionic strength and/or pH-dependent dissociation into smaller subunits [4,5]. This latter group of methods yields the H_3–H_4 tetramer and the H_{2A}–H_{2B} dimer.

For the reconstitution of histone–histone and histone–DNA complexes it is in many cases desirable to have individual polypeptide chains available. Such histones can only be prepared efficiently by either one or a combination of several of the methods in the first group. It therefore becomes important to establish whether histone polypeptide chains isolated by such methods can be renatured to result in high yields of natural histone–histone complexes.

We wish to report that the direct reconstitution in good yields of a stable H_3–H_4 tetramer from acid extracted and lyophilized histones requires the presence of all four core histones. Such a tetramer appears identical to the natural tetramer on the basis of its physico-chemical properties.

2. MATERIALS AND METHODS

2.1. *Histone purification*

The natural H_3–H_4 tetramer of chicken erythrocyte chromatin was prepared essentially as described previously except that the exclusion chromatography used to separate H_3–H_4 tetramer from H_3–H_4 dimer and H_{2A}–H_{2B} dimer was carried out at 150 mM NaCl, 50 mM phosphate (pH 7.4) instead of 2 M NaCl, 50 mM acetate (pH 5.0)

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to avoid proteolysis. Under these conditions the aspartate protease present in chicken chromatin is inactive [6]. The H_3–H_4 tetramer containing fractions were pooled and the tetramer precipitated with 0.7 saturation ammonium sulphate. H_1 and H_5 contamination is removed by this step since these histones are soluble at 0.7 saturation ammonium sulphate [4]. Individual acid denatured histones H_3 and H_4 were purified as described previously [3] the purification of H_3 involved dimerisation with subsequent reduction [7]. Histone H_3 prepared in this way is virtually free from dimer, and the H_4 free of other contaminating histones. An equimolar mixture of core histones was prepared by perchloric acid precipitation of total acid extracted histones [8]. The precipitated core histones were taken up in 0.25 M HCl, dialysed against 0.25 M HCl, water, and freeze dried.

2.2. *Spectroscopy*

Fluorescence spectroscopy was carried out on an Aminco SPF 500 corrected spectra spectrofluorometer.

2.3. *Ultracentrifugation*

A Beckman Model E Ultracentrifuge, equipped with Schlieren Optics, was used.

2.4. *Crosslinking*

Histone crosslinking was carried out with a bifunctional imidoester in 50 mM phosphate 150 mM NaCl (pH 8.0). Aliquots were then subjected to SDS electrophoresis [11] on a 20% polyacrylamide slab gel. Further experimental details are in the legend to fig.4.

3. RESULTS

As has been previously reported [9,10] acid extracted histones H_3 and H_4 interact in low ionic strength at pH 7 to form a complex as determined by the unchanged intrinsic fluorescence polarisation (fig.1). However, the size of the complex estimated by its appearance in the exclusion volume of a molecular sieve column (fig.2) and its *S*-value (table 1) far exceeds that of the H_3–H_4 tetramer at a concentration of 3 mg/ml.

However, if a mixture of all five acid-extracted, lyophilized histones (or of the core histones only) is dissolved in 2 M NaCl at pH 7 and subsequently the salt concentration is lowered to 150 mM a histone complex of tetrameric size is produced (fig.2). A complex of apparently tetrameric size is also produced from a mixture of all acid extracted lyophilized histones dissolved in 150 mM NaCl at pH 7. Both these complexes form from a lyophilized mixture of either total or core histones only. The S values of all the complexes (table 1) correspond to their tetrameric and polymeric nature respectively.

Table 1

Sedimentation velocities of histone complexes

Complex	*S*-value
H_3–H_4 aggregate by addition of equimolar amounts of H_3 and H_4	>20 S
H_3–H_4 aggregate from total acid extracted histones via 2 M NaCl	>20 S
via 0.15 M NaCl	>20 S
H_3–H_4 tetramer from total acid extracted histones via 2 M NaCl	2.78 S
via 0.15 M NaCl	2.81 S
H_3–H_4 tetramer by salt extraction method of [5]	2.75 S

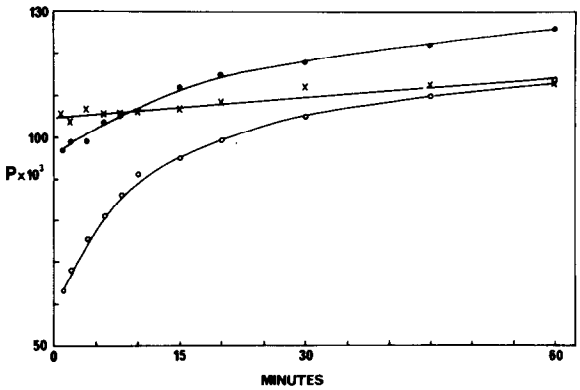

Fig.1. Intrinsic fluorescence polarisation changes due to the addition of an equal volume of 100 mM phosphate buffer (pH 7.4) containing 0.3 M NaCl to solutions of acid purified histones in water. (•—•)H3; (○—○)H4; (×—×) equimolar mixture of H_3 and H_4. Histone concentration 10^{-5} M. Excitation wavelength 259 nm, emission wavelength 310 nm.

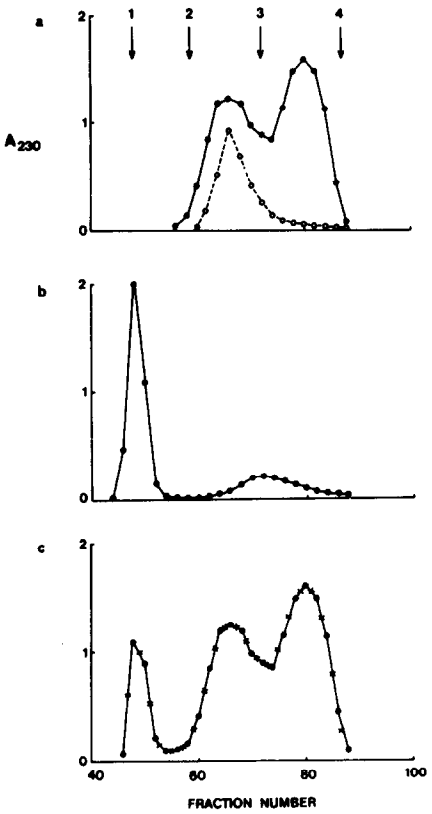

Fig.2. Sephadex G-100 gel filtration of histone complexes. The same column (1 m × 2.5 cm) was used throughout. Histone samples applied to the column had a concentration of between 10–12 mg/ml. Buffer: 150 mM NaCl, 50 mM phosphate (pH 7.4). The arrows denote the position of elution (peak tube) of molecular weight markers used: 1, Blue Dextran; 2, bovine albumin; 3, ovalbumin; 4, myoglobin. (a) Chromatography of histones extracted from chromatin by 2 M NaCl (•—•). The H_3–H_4 tetramer peak was pooled, precipitated with 0.7 saturation ammonium sulphate and rerun (○—○). (b) Chromatography of the aggregate formed by simple mixing of acid purified H_3 and H_4. (c) Chromatography of high (•—•) or low (×—×) salt reconstituted histones.

The composition of the complexes becomes apparent on SDS–polyacrylamide gel electrophoresis (fig.3). The high molecular weight aggregates in the exclusion volume are aggregates of the histones H_3 and H_4; the tetrameric fraction contains H_3 and H_4. Depending on whether the starting material consisted of core histones or total histones, the tetrameric fraction also contained the coeluted H_1 and H_5. The subsequent dimeric fraction contained the histones H_{2A} and H_{2B}.

The aggregates formed from acid extracted lyophilized histone H_3 and H_4 on dissolving in 150 mM NaCl at pH 7 and the two types of tetramer form distinctly different crosslinked products on treatment with dimethyl-3,3′-dithiobis-propionimidate (fig.4). The aggregate proceeds quickly via the different dimer types to *n*-mers without significant accumulation of tetramers. The two tetramers, reconstituted at high or low ionic strength in contrast form mainly heterotypic dimer before transition into a tetramer with subsequent *n*-mer formation. That the tetramer formed is in fact heterotypic is evident from fig.5. After cleavage of the crosslinking reagent the 2nd dimension of the slab gel clearly identifies the histones H_3 and H_4.

If the intrinsic fluorescence of the histones H_3 and H_4, due to their tyrosine residues is taken as a measure of identity of the tyrosine environment in

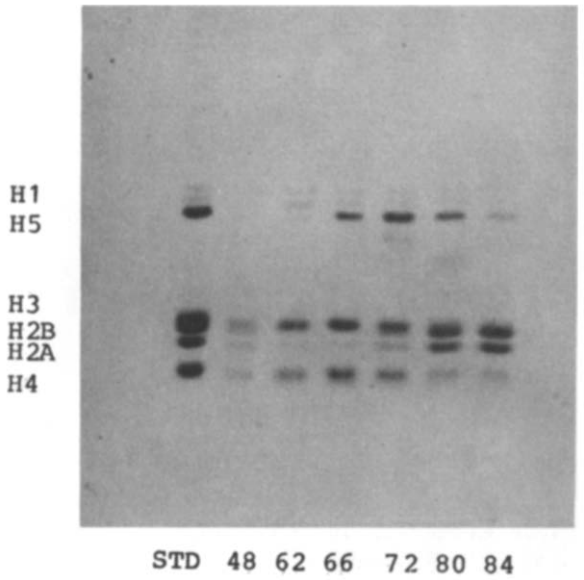

Fig.3. SDS–polyacrylamide gel electrophoresis of histones separated by Sephadex G-100 gel filtration (fig.2). STD refers to total chicken erythrocyte acid extracted histones.

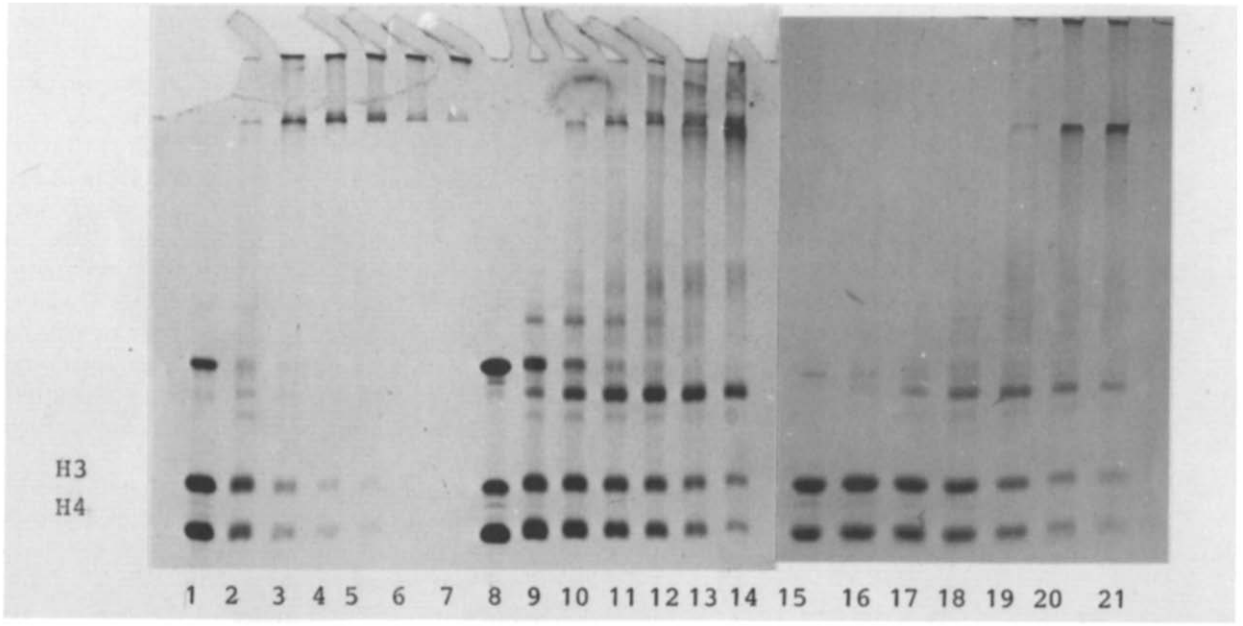

Fig.4. SDS-polyacrylamide gel electrophoretogram of 3,3′-dimethyl-dithiobispropionimidate crosslinking of histones in 50 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. The reaction was stopped by the addition of excess glycine. Lanes 1–7: crosslinking of the aggregate formed by simple mixing of acid purified H_3 and H_4 stopped at 0,2,5,10, 20, 40 and 60 min respectively; lanes 8–14: ditto except salt extracted H_3–H_4 tetramer; lanes 15–21 high salt reconstituted tetramer; lane 11 was excised from a similar gel before staining and rerun on another 2-dimensional gel (fig. 5) to confirm that the tetrameric region consisted of equivalent quantities of H_3 and H_4.

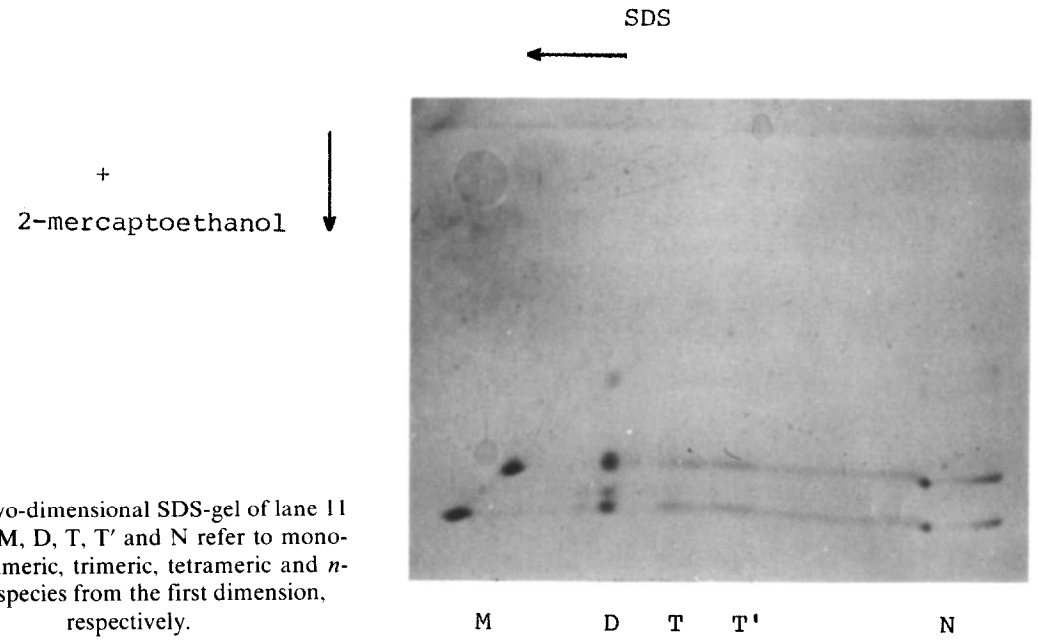

Fig.5. Two-dimensional SDS-gel of lane 11 of fig.4. M, D, T, T′ and N refer to monomeric, dimeric, trimeric, tetrameric and *n*-meric species from the first dimension, respectively.

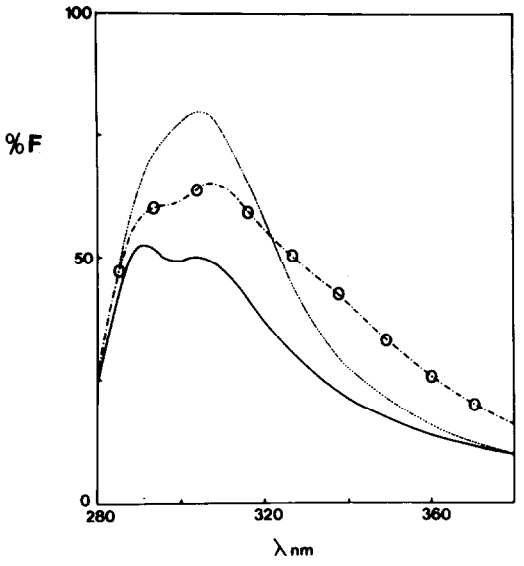

Fig.6. Fluorescence emission spectra of the aggregate formed by simple mixing of acid purified H_3 and H_4 (—), the salt extracted tetramer (-·-·-·), the high reconstituted tetramer (○-·-·-·○) and the low reconstituted tetramer (·····). Protein concentration 0.1 mg/ml. Excitation wavelength 259 nm.

the tetramers, then the H_3–H_4 tetramer reconstituted at 2 M NaCl is identical to the natural tetramer extracted from chromatin (fig.6). In the tetramer reconstituted at 150 mM salt from acid extracted lyophilized total histones the tyrosine environment is different as is the tyrosine environment in the H_3–H_4 aggregate. Similarly the CD spectra of the natural tetramer and the one reconstituted at high salt are identical but different from that of the H_3–H_4 aggregate (fig.7).

4. DISCUSSION

Controversy exists as to whether the denaturing conditions employed in the purification of histones lead to irreversible changes in their conformation. Hydrodynamic [11], electrophoretic [12] and spectroscopic evidence [9] has been provided to show that the degree of denaturation may be only slight and easily reversible. Alternatively irreversible reduction of helix content [13] changes in the dimer–tetramer equilibrium [14] and formation of

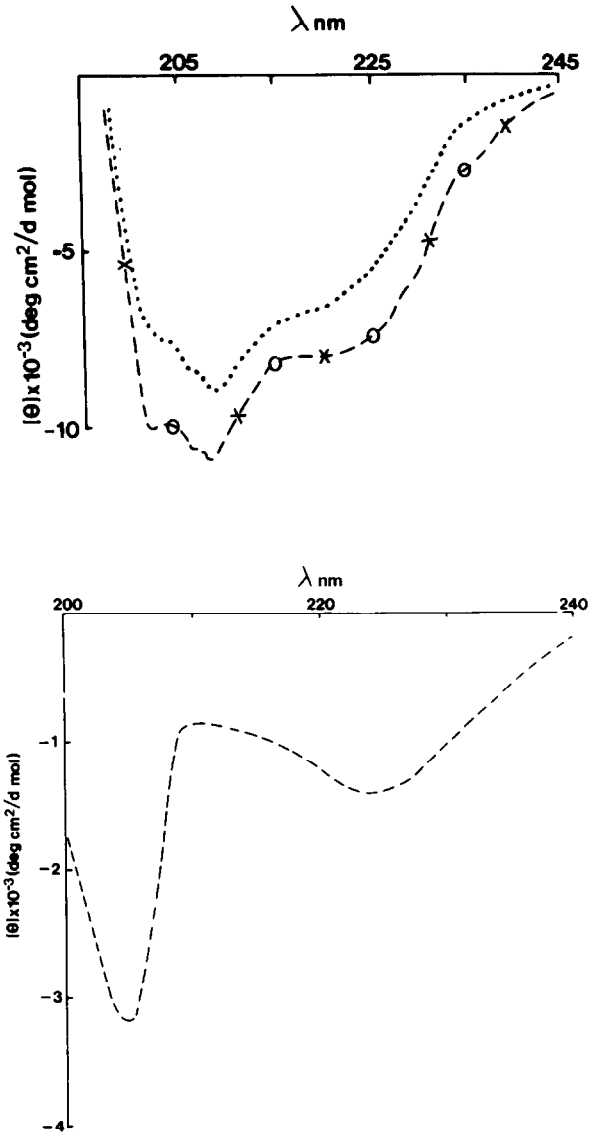

Fig.7. (a) Circular dichroism spectra. Aggregate formed by simple mixing of acid purified H_3 and H_4 (·····), salt extracted H_3–H_4 tetramer and high (○------○) and low (×-----×) salt reconstituted tetramer (-------). Protein concentration: 2 mg/ml, cell path length: 0.1 mm, full scale: 0.02 degrees. (b) CD difference spectrum.

high molecular weight aggregates [15] have been reported. The typical X-ray diffraction pattern characterising the repeat structure of chromatin

could only be produced after reassembly from salt extracted histones [15]. Though core particle like structures have been prepared from acid extracted histones [16], the yield of such particles cannot be gleaned from the reports.

In the reconstitution experiment reported here the histone concentration is between 100–200 times larger than used previously in the literature [9,10]. Under the present conditions only the salt extracted natural H_3–H_4 complex exists solely in the form of the tetramer, the acid extracted H_3 and H_4 histones form an aggregate larger than 20 S. When however a mixture of all the acid extracted core histones (or total histones) are exposed first to conditions known to allow octamer formation and then subsequently subjected to a decrease in the salt concentration, formation of a H_3–H_4 tetramer occurs though still approximately 20% of the histones H_3 and H_4 aggregate to a size larger than 20 S. This aggregation may well be the result of misdirected protein–protein interactions. The process of conformational change from the random coil in acid to the ordered structure in high salt leads to the presentation of sites responsible for tetramer formation and for interaction with other histones. In the absence of histone H_{2A} and H_{2B} these latter sites apparently also interact with additional histones H_3 and H_4 to yield an H_3–H_4 aggregate. When histones H_{2A} and H_{2B} are present competition between the histones for such sites presumably yields the octamer which subsequently is dissociated into dimers and a tetramer stable at low ionic strength.

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