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Physical Studies on the H3/H4 Histone Tetramer[†]

Tom Moss,* Peter D. Cary, Colyn Crane-Robinson, and E. M. Bradbury

ABSTRACT: High-resolution proton magnetic resonance spectroscopy (270 MHz), circular dichroism, and infrared spectroscopies and ultracentrifugation studies have been carried out on the salt-extracted $(H3/H4)_2$ tetramer from calf thymus. The tetramer contains about 29% α helix and no β structure. It is denatured in 6 M urea but can be renatured simply by dialysis to water. The proton spectrum shows a number of perturbed resonances which are not observed in the spectra of either H3 or H4 alone. The observation of these resonances demonstrates that the tetramer contains some elements of tertiary structure. The overall appearance of the spectrum however is close to that of a partially denatured protein. Sedimentation velocity studies show the tetramer to have a frictional ratio of 1.99 in 50 mM acetate/50 mM bisulfite and thus to be hydrodynamically quite different from a globular protein. Two possible structural models compatible with the data are discussed.

Much recent work has suggested a subunit structure for chromatin in which the four histones H2A, H2B, H3, and H4[1] are complexed with about 200 base pairs of DNA to form a unit of mass about 250 000 daltons. On salt dissociation of chromatin the last histones to be removed are the arginine-rich pair H3 and H4 which are both known to have very highly conserved sequences. Evidence that this pair of histones can form a structural unit has come from cross-linking studies of both salt dissociated histone and of chromatin itself. The conclusion drawn from this work (Kornberg and Thomas, 1974) (Thomas and Kornberg, 1975) was that the unit of structure was a tetrameric $(H3/H4)_2$. The molecular weight of the salt-dissociated H3/H4 complex has been studied by Roark et al. (1974) using sedimentation equilibrium and making a specific correction for primary charge effects. They obtained a molecular weight of 54 000 which is close to the value calculated for an $(H3/H4)_2$ tetramer. At low concentration a significant reduction in the apparent molecular weight was interpreted as a consequence of an equilibrium between tetrameric and dimeric forms. More recently, D'Anna and Isenberg (1974a) demonstrated that, by reassociation of an equimolar ratio of acid-extracted H3 and H4, a complex of apparent molecular weight 54 000 could be obtained. This observation was important since Kornberg and Thomas had suggested that acid extraction of histones irreversibly denatures their native structure. The observations described above indicated the chemical composition and molecular weight of the complex, but no details of secondary or tertiary structure.

D'Anna and Isenberg (1974b) using reconstituted material showed that the complex contained about 25% α helix and apparently no β structure. This helix content is not dissimilar to the average of the separated histones, but the absence of β structure at high ionic strength is characteristic of the complex. The only possible indicators of tertiary structure so far published are the observation of Kornberg and Thomas (1974) that the complex shows a sedimentation coefficient of 3 S, corresponding to a frictional ratio of 1.7, and the observation that the histone tetramer elutes at an apparent molecular weight of ~100 000 from Sephadex G-100 (van der Westhuyzen and Von Holt, 1971). These observations imply that the tetramer is not a fully compact globular structure.

In view of the foregoing, we have undertaken a more detailed investigation of the secondary and tertiary structure of the H3/H4 complex, using circular dichroism (CD), infrared (ir), and high-resolution proton magnetic resonance spectroscopy (^{1}H NMR).[2] The last technique is a particularly sensitive indicator of compact tertiary structure since the close packing of residues in the inner parts of globular regions gives rise to perturbed resonances. The effect is most marked if aromatic

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[†] From the Department of Physics, Biophysics Laboratory, Portsmouth Polytechnic, Portsmouth, Hants., United Kingdom. *Received August 19, 1975.* This research was supported by the Science Research Council of Great Britain.

[1] The new histone nomenclature used here was accepted by the participants at the CIBA Foundation Symposium on Structure and Function of Chromatin, April 3-5, 1974. This new nomenclature, which has been proposed to the appropriate international nomenclature committee, is as follows for each histone where the previous names are given in parentheses: H2A (F2A2, IIb1, ALK), H2B (F2B, IIb2, KSA), H3 (F3, III, ARK), H4 (F2A1, IV, GRK), and H5 (F2C, V, KAS).

[2] Abbreviations used are: ^{1}H NMR, proton magnetic resonance; DSS, sodium 2,2-dimethyl-2-silapentanesulfonate; CD, circular dichroism; DTT, dithiothreitol.

residues are involved, when the so-called "ring-current shifted" peaks are observed. In addition to these spectroscopic techniques, we have also made a study of the concentration dependence of the sedimentation coefficient as an indicator of the shape and size of the complex.

The concept that the H3/H4 tetramer is a fully globular complex accords with the hypothesis of Kornberg and Thomas that the histones form a solid compact protein core on the outside of which is wound the DNA, and they have proposed a roughly spherical H3/H4 complex of diameter 40-50 Å (Kornberg, 1974). Since the H3/H4 complex appears to be a fundamental building block of the chromatin subunit, it is of prime importance to establish whether it is indeed fully globular in solution and roughly spherical or whether it departs significantly from this in either shape or size.

Experimental Section

Histone Isolation. Chromatin was prepared from freshly excised calf thymus by the method of Panyim et al. (1971). Following the procedures of van der Westhuyzen and Von Holt (1971), the histones were extracted from the chromatin in 2 M NaCl, 50 mM sodium acetate buffer, 50 mM sodium bisulfite, pH 5.0. The DNA was then precipitated by the addition of protamine (Sigma Grade 1) and dialysis to 0.15 M NaCl in the above buffer. The histones so extracted were separated from excess protamine on Sephadex G-50 and fractionated on Sephadex G-100. Addition of ammonium sulfate to the G-100 peak containing H3, H4, and H1 precipitated a mixture of the H3 and H4 histone fractions, leaving H1 in solution.

The purity of the H3/H4 histone mixture produced by the above procedure was found to be greater than 95% on disc gel electrophoresis (Panyim and Chalkley (1969)).

Amino acid analyses of the H3/H4 histone mixture, making no correction for hydrolytic losses in a hydrolysis time of 20 h, agreed within experimental error with the expected amino acid composition of an equimolar H3/H4 mixture (De Lange et al., 1969; Ogawa et al., 1969; De Lange et al., 1973).

Separated H3 and H4 histone fractions used in comparison studies were prepared by the procedures of Johns (1964, 1967) and Oliver et al. (1972). They were greater than 98% pure as checked by disc gel electrophoresis.

Although the present studies have been repeated on several different batches of H3/H4 histone, the majority of the data given here were obtained on a single preparation. The protein concentration used was of the same order for all the techniques employed except ultracentrifugation for which a range of concentrations was studied.

High-Resolution Proton Magnetic Resonance Spectroscopy (^1H NMR). ^{1}H NMR spectra were recorded at known histone concentration (in the region of 0.5 mM $(H3/H4)_1$ (i.e. about 13 mg/ml)) at 18 °C, on a Bruker 270-MHz Fourier transform NMR spectrometer. All spectra were referenced for area against 5% trifluoroacetic acid (w/w) in carbon tetrachloride contained in an insert tube. Chemical shifts quoted are from sodium 4,4-dimethyl-4-silapentanesulfonate (DSS). Convolution difference spectra were obtained by the method of Campbell et al. (1973). This is a method of mathematically reducing line widths in a spectrum by subtracting from that spectrum an artificially broadened version of the same spectrum.

Circular Dichroism. CD spectra were recorded at known concentrations in the region of 0.25 mM $(H3/H4)_1$ (~7 mg/ml) at room temperature on a Cary 61 spectropolarimeter.

Concentrations were determined from uv spectra using the mean of the extinction coefficient at 275 nm for histones H3 and H4, taken as 4.04×10^3/mol and 5.40×10^3/mol, respectively.

Estimates of α helicity were made at 222 nm assuming a random coil ellipticity of −500°, a 100% α-helical ellipticity of −30 000°, and a linear relationship between $[\theta]_{222}$ and α-helix content (unpublished results of this laboratory, and Chen et al. (1974)).

Infrared Spectroscopy. Ir spectra were recorded at approximately 0.25 mM $(H3/H4)_1$ (~7 mg/ml) at room temperature on a Grubb Parsons Spectromaster using a 75-μm path length. The resolution of this instrument is ~3 cm^{-1} in the 6-μm spectral region.

Ultracentrifuge Experiments. Sedimentation velocity experiments were performed at various histone concentrations in an MSE Centriscan ultracentrifuge at a speed of 54 000 rpm and a temperature of 20 ± 0.25 °C using scanning absorption optics.

A high-speed sedimentation equilibrium experiment was performed (Yphantis, 1964) at 0.1 mM $(H3/H4)_1$ (~2.5 mg/ml), loading concentration in a Beckman Spinco Model E ultracentrifuge at a speed of 34 000 rpm using interference optics.

Results and Discussion

The H3/H4 equimolar histone mixture, prepared by the methods described above, was studied after each of three different pretreatments.

(1) The "native material": prepared from the ammonium sulfate precipitate by dissolution and thorough dialysis at 4 °C against 50 mM sodium acetate buffer, 50 mM sodium bisulfite, pH 5.0. Deuterated salts in D_2O were used for ^{1}H NMR and ir measurements.

(2) The "previously denatured" material: prepared from the ammonium sulfate precipitate by dissolution in 7 M urea, 10 mM HCl, followed by dialysis against the solvent for more than 20 h. This was then followed by exhaustive dialysis against H_2O or D_2O + 0.1 mg/ml of dithiothreitol (DTT).

(3) The "renatured" material: prepared as in (2), but this was then followed by dialysis against 50 mM sodium acetate buffer and sodium bisulfite, pH 5.0. A few samples were prepared by straight addition of sodium acetate/bisulfite to "previously denatured" samples, but no differences were observed between samples from the different procedures. Ultracentrifuge samples of "renatured" material were always prepared, however, by dialysis.

Samples were checked after the experiments for degradation and H3 dimerization by polyacrylamide gel electrophoresis (Panyim and Chalkley (1969)). Degradation was found to be less than 5% even after 72 h at 20 °C. H3 dimerization was found to be less than 5% under the same conditions.

^1H NMR of the "Native" Histone. Figure 1 shows spectra of "native" H3/H4 histone and also of "native" samples after further addition of 100 mM NaCl. These are solution conditions under which the spectra of the pure histone fractions show apparent loss of area due to the extreme spectral broadening which occurs on self-aggregation. For example, at an ionic strength of ~0.2 both H3 and H4 spectra show more than 80% loss of apparent area. The areas of the well-defined spectra of Figure 1 account for at least 70% of the histone in solution, as will be shown in the ultracentrifugation and denaturation ^{1}H NMR studies. This demonstrates that at these concentrations the strong aggregation properties of the separated histones are largely inhibited in the mixture, as has been concluded from ultracentrifugation studies by Kornberg and Thomas (1974), by Roark et al. (1974), and from light scattering studies by

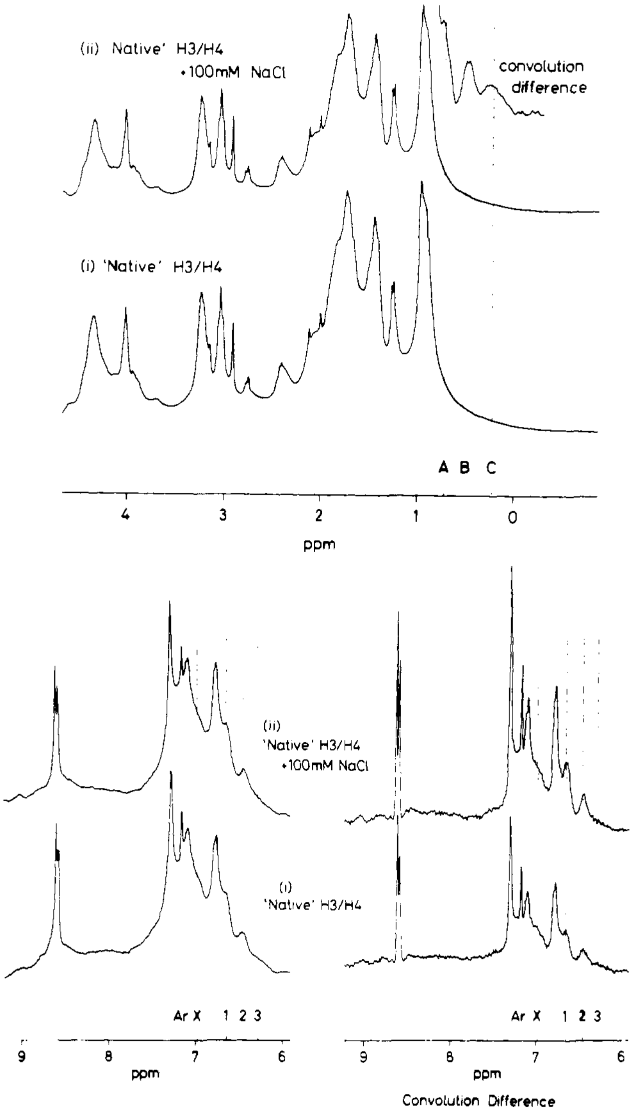

FIGURE 1: (a) Upfield (top), (b) downfield (bottom), 270-MHz ^{1}H NMR spectra of the "native" H3/H4 histone at ~0.5 mM H3/H4: (i) 50 mM sodium acetate-d_3, 50 mM sodium bisulfite-d, pH 4.8; (ii) +100 mM NaCl, pH 4.8, added as concentrated NaCl in the above buffer.

D'Anna and Isenberg (1974b).

The most striking feature of the spectra in Figure 1 is that they bear very little resemblance to the spectra of globular proteins. The latter are characterized by a given type of amino acid showing a range of chemical shifts due to the spread of microenvironments through the tertiary structure. The total spectrum envelope is thus the sum of many partially, but not fully, overlapping resonances. In a fully denaturated protein, residues of a given type become equivalent and the spectrum simplifies to a reduced number of better defined resonances. The apparent resolution in a denatured protein is further enhanced by the fact that the individual line widths are themselves significantly less than in the native globular state. These differences are illustrated in Figure 2 for bovine serum albumin (a protein of molecular weight comparable to that of the (H3/H4) complex) that is partially, but not fully, denatured at pH 2 and is fully denatured in 8 M urea. It is immediately apparent that the spectra of the H3/H4 complex are similar in general appearance to that of partially denatured bovine serum albumin, indicating that, under the experimental conditions used here, the conformation of the H3/H4 complex is very far from that of a compact globule.

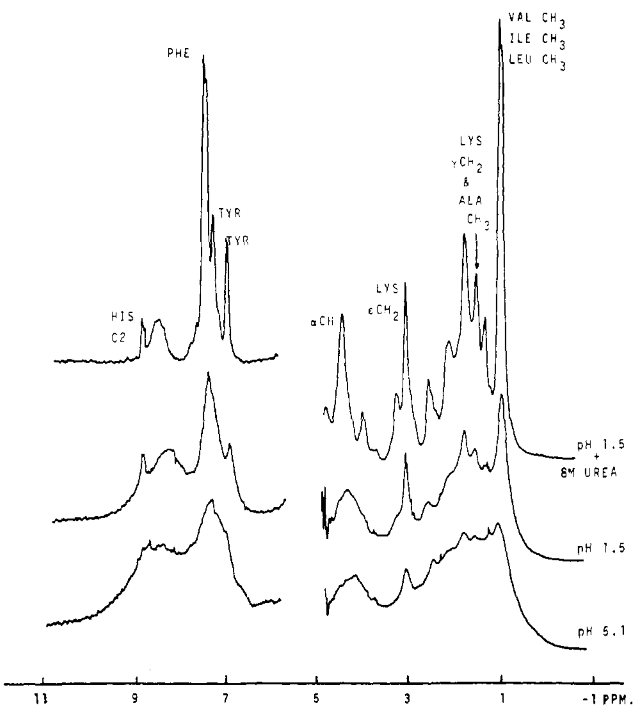

FIGURE 2: Spectra, 270-MHz, of bovine serum albumin (mol wt 66 500) at 10 mg/ml, 20 °C: pH 5.1 (20 mM acetate buffer, 0.15 M NaCl), native; pH 1.5 (DCl added to pH 5.1 solution), partially denatured; and pH 1.5 + 8 M urea, fully denatured.

Close inspection of the spectra of the complex (Figure 1) shows the presence of both ring-current shifted peaks (between 0 and 1 ppm) and of perturbed aromatic resonances, in addition to the peaks at chemical shifts characteristic of the random coil state. These perturbed resonances are visualized better in the convolution difference spectra. The upper spectrum is included in Figure 1 since it was noted that addition of 100 mM NaCl to the "native" material caused the perturbed resonances to sharpen somewhat. Such perturbed resonances are caused by close contacts between different chemical groups of the protein. In the special case of ring current shifted resonances, one of these groups is aromatic. The observation of these perturbed resonances demonstrates the formation of some specific tertiary structure in the H3/H4 histone.

It is not at present possible to give any assignment of the perturbed resonances shown in Figure 1 and, therefore, it is not known whether they arise from specific H3/H4 intermolecular interactions or from intramolecular folding of the individual molecules, although the latter is more likely. It is important to note that the aggregation of pure H3 and H4, and consequent line broadening, makes it impossible to obtain evidence of tertiary structure, if that exists, with the pure histones. It could therefore be that the lack of aggregation in the H3/H4 histone simply allows the ^{1}H NMR observation of specific tertiary structure which is also present in the aggregated state of the pure histone fractions. The segments of the histone chains involved in self-aggregation are in general those which are rich in apolar residues (Bradbury and Rattle, 1972; Bradbury et al., 1973). These segments are, therefore, those most likely to be involved in the specific tertiary structure of the H3/H4 histone.

The effect of sequential addition of NaCl to the "native" H3/H4 histone can be seen in Figure 3. Beyond 300 mM added NaCl loss of area is noted, indicating the onset of large scale aggregation. The spectral changes are similar to those

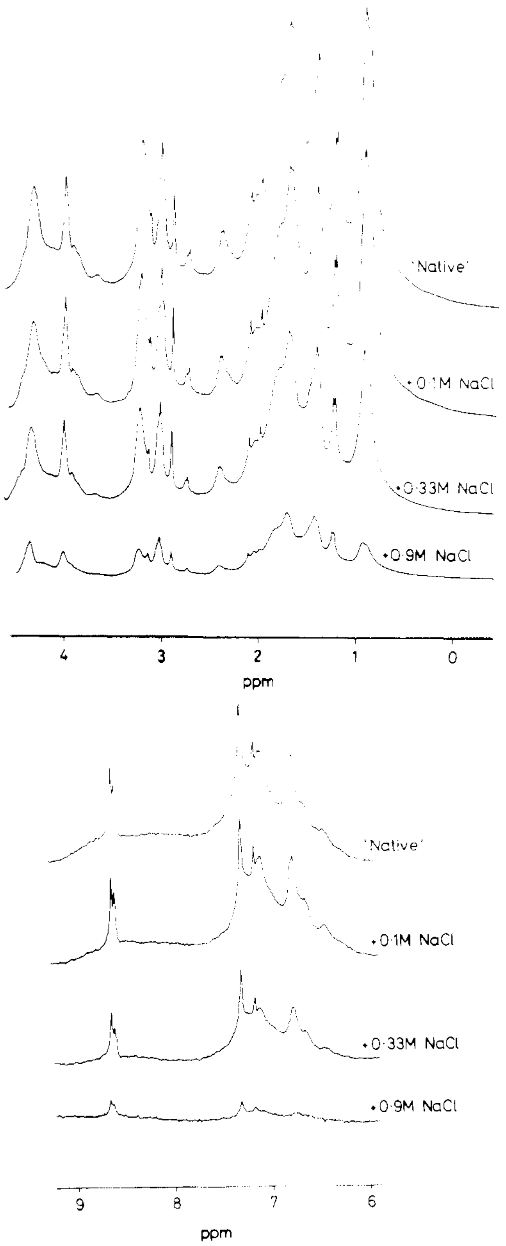

FIGURE 3: (a) Upfield (top), (b) downfield (bottom), 270-MHz ^{1}H NMR spectra of salt-induced changes in the native H3/H4 histone at ~0.5 mM $(H3/H4)_1$ (initially in 50 mM sodium acetate-d_3, 50 mM sodium bisulfite, pH 4.8). A concentrated NaCl solution in the above buffer was added to the sample in appropriate aliquots to obtain the final NaCl concentrations shown in the figure.

noted with the separated histones at very much lower ionic strength.

Increasing the pH of the "native" histone from pH 5 in steps of ~0.5 to above pH 7 by the addition of NaOH did not cause any significant changes in the positions or intensities of the perturbed resonances referred to above.

The details of the ^{1}H NMR spectra will be considered further, after discussion of the data obtained with the "previously denatured" and "renatured" samples.

CD and Ir of the "Native" Histone. Figure 4 shows the CD spectra of the native histone (ii) and that in 1 M NaCl (i). The α-helix content of the native histone, as estimated from constants given above, is 29 ± 2%, rising to 35 ± 2% in 1 M NaCl.

The CD spectra of pure H3 and H4 were also obtained at 0.25 mM under the same conditions as that of the native H3/H4 histone. The average of the α-helix content of these two histones was found to be 28 ± 6%, i.e., within experimental error the same as that of the native histone. Although the estimates of helicity in the pure H3 and H4 solutions are less accurate due to the presence of β structure, we do not find experimentally significant differences in helicity between the interacting and noninteracting states. D'Anna and Isenberg (1974), however, find a significant difference between the two states. Our results were obtained under somewhat different conditions, in particular at 25 times the histone concentration, and are, therefore, not strictly comparable. These discrepancies require further study.

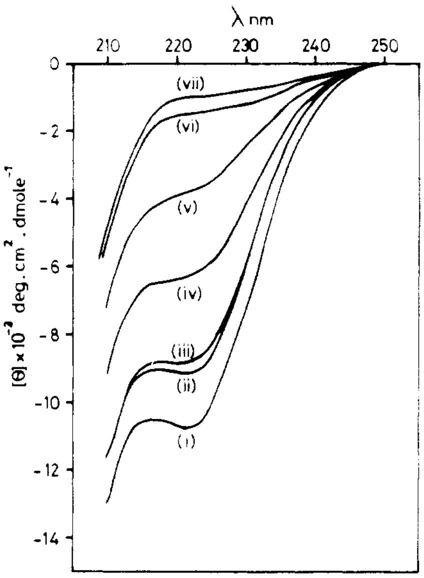

FIGURE 4: CD spectra of the H3/H4 histone at ~0.25 mM $(H3/H4)_1$. (i) Native (50 mM sodium acetate, 50 mM sodium bisulfite, pH 5.0) + 1 M NaCl, added as concentrated NaCl in buffer; (ii) native; (iii) previously denatured (H_2O/0.1 mg/ml DTT); (iv) previously denatured, 1.2 M urea, pH 2.0; (v) previously denatured, 2.7 M urea, pH 2.0; (vi) previously denatured, 6.0 M urea, pH 2.0; (vii) previously denatured, 6.0 M urea, pH 8.0. Urea was added as appropriate aliquots of a concentrated solution containing 0.1 mg/ml of DTT, except in the case of 6 M where solid urea was added.

The ir spectrum of the native histone was also recorded since the amide I vibration at ~6 μm is very sensitive to the formation of β structure. No characteristic β band at ~1610 cm^{-1} was observed either in 50 mM acetate/bisulfite, or even after the addition of 1 M NaCl and leaving the sample at room temperature for 24 h. This is in marked contrast to the separated H3 and H4 histones, which both show ~30% β structure in high salt (D'Anna and Isenberg, 1974b; Lewis et al., 1975).

Ultracentrifugation of the Native Histone. Sedimentation velocity experiments were performed at various concentrations of the native H3/H4 histone. A well-defined histone–solvent boundary was always obtained, of which a typical example is shown in Figure 5 (inset). The corrected sedimentation coefficient, $s_{20/w}$, was calculated from the velocity of the inflexion point of the boundary and plotted against histone concentration (Figure 5). Extrapolation of this plot to infinite dilution, using a linear least-squares fit to the data, gave a sedimentation constant, $s_{20,w}{}^0$, of 2.54 ± 0.05 S.

It was found that 20–30% of the histone loaded in a sedimentation velocity experiment sedimented at more than 20 S. This was assumed to be a nonspecific histone aggregate. No material was observed to sediment at s values between 2.5 and 20 S. A single high-speed sedimentation equilibrium experiment was performed on the native H3/H4 histone at a loading

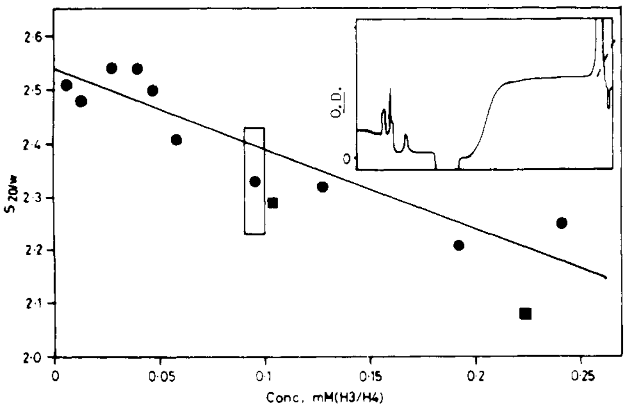

FIGURE 5: Plot of $s_{20/w}$ against protein concentration for the (●) native H3/H4 histone and (■) renatured H3/H4 histone both in 50 mM sodium acetate, 50 mM sodium bisulfite, pH 5.0. The extrapolation to infinite dilution was made by a linear least-squares fit to the native histone data. The inset shows a typical optical density scan of the sedimenting boundary.

concentration of 0.1 mM $(H3/H4)_1 \sim 2.5$ mg/ml. The molecular weight obtained was 41 000 ± 1000 within the concentration range 0.003–0.023 mM $(H3/H4)_1$, making no correction for primary charge effects. This is in reasonable accord with the corrected data of Roark et al. in this concentration range (~46 000). This measurement gives an assurance that the present work has been performed on the same state of the H3/H4 histone as studied by the above authors.

An equilibrium constant of 1.98×10^5 l./mol was calculated by Roark et al. for the H3/H4 $\rightleftarrows$ $(H3/H4)_2$ associating system. The work of Gilbert and Gilbert (1973) indicates that with this equilibrium constant the association would be detected in the concentration range of the $s_{20/w}$ plot of Figure 5, as an initial increase, followed by a substantial decrease in apparent sedimentation coefficient with decreasing concentration. Reference to Figure 5 will show that this was not, however, observed.

Assuming a molecular weight of 54 000 for the tetramer and a partial specific volume of 0.733 ml g^{-1} (estimated from the known amino acid compositions using the data of Cohn and Edsall (1941)) a frictional coefficient (f/f_o) of 1.99 ± 0.04 was calculated for the tetramer. This value is vastly in excess of that observed for globular proteins, and it is concluded that the structure is either far more expanded than that of the usual globular proteins or is relatively compact but very asymmetric.

^{1}H NMR of the Previously Denatured and Renatured Histone. The ^{1}H NMR spectrum of the previously denatured H3/H4 histone in D_2O/DTT at pH 5.3 can be seen in Figure 6 (iii). Comparison of this spectrum with that of the native histone (Figure 6 i) shows that it contains all of the shifted resonances characteristic of the native H3/H4 interaction. It also was found that addition of 10 mM DCl to the previously denatured histone in water caused no significant changes in the ^{1}H NMR spectrum.

The effect on the ^{1}H NMR spectrum of the addition of urea to the previously denatured histone at pH 2.0 can be seen in Figure 6 (iv) and 6 (v). By 6 M urea (Figure 6v), all of the aromatic shifted resonances and the ring current shifted resonances have disappeared. It was noticed that there was a 20–30% increase in spectral area in 6 M urea over that of the native histone. This corresponds to the 20–30% aggregated material noticed in the ultracentrifuge experiments. Partial denaturation is obtained in 1 M urea (see Figure 6iv, in which ring current C has disappeared and the aromatic shifted resonances have decreased in area and also shifted to lower field

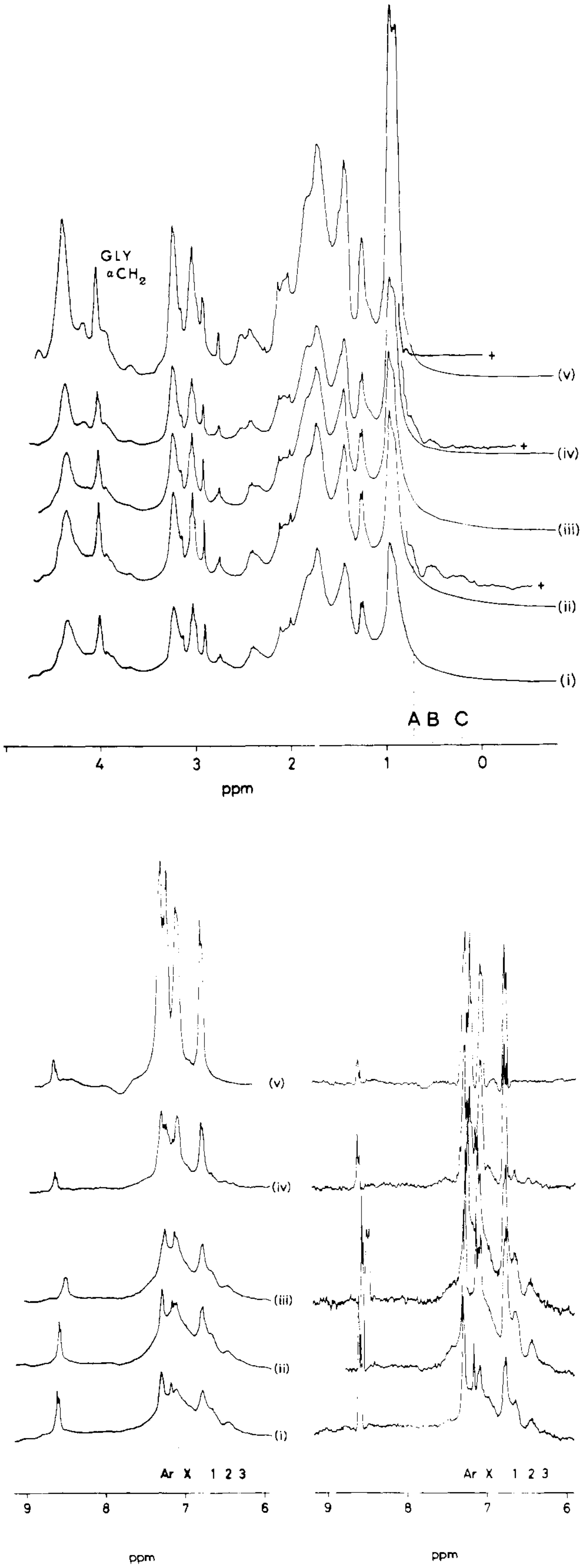

FIGURE 6: (a) Upfield (top), (b) downfield (bottom), 270-MHz ^{1}H NMR spectra of the H3/H4 histone under various conditions: (i) native; (ii) renatured (25 mM sodium acetate-d_3, 25 mM sodium bisulfite-d, pH 5.0); (iii) previously denatured in D_2O (0.1 mg/ml of DTT) pH 5.3; (iv) previously denatured in D_2O (0.1 mg/ml of DTT) pH 2, 1 M urea; (v) previously denatured (0.1 mg/ml of DTT), pH 2, 6 M urea. (+) Convolution difference.

TABLE I: Analysis of Aromatic ^{1}H NMR Spectral Areas.

Resonance peak	Tyr ortho	1	2	3
No. of protons per (H3/H4) pair	12	5	3	1.5

slightly).

The ^{1}H NMR spectrum of the renatured H3/H4 histone (Figure 6, ii), also contains all of the shifted resonances characteristic of the native H3/H4 interaction.

CD of the Previously Denatured Histone. The CD spectra of the previously denatured H3/H4 histone in H_2O/DTT, pH 5.0, and on the addition of urea can be seen in Figure 4.

From this figure it can be seen that the native and previously denatured CD spectra show a difference in α-helix content of only a few percent. Figure 4 also shows that the $[\theta]_{222}$ parameter of the previously denatured histone decreases successively with increases in urea concentration, reaching a minimum of −1000° in 6 M urea.

Ultracentrifugation of the Renatured Histone. Sedimentation velocity experiments were performed on the renatured H3/H4 histone in 50 mM sodium acetate/bisulfite, pH 5.0. The corrected sedimentation coefficients, $s_{20/w}$, so obtained lie within experimental error on the plot of $s_{20/w}$ against concentration for the native histone (see Figure 5).

It was noted that approximately 20% of the histone loaded in these experiments sedimented at greater than 20 S.

Analysis of the ^{1}H NMR Spectra. A comparison of the areas of the aromatic resonances in the ^{1}H NMR spectra of the native, previously denatured, and renatured H3/H4 histone has been made in order to have some assessment of the number of perturbed protons. For all three preparations the results were the same within the experimental error. The histidine C-2 and C-4 proton resonances are unreliable in area due to their relatively long relaxation time and their tendency to become deuterated (see, for example, the spectrum in 6 M urea, pH 2, Figure 6v). The area of the C-2-H peak at 8.7 ppm was therefore subtracted from the total area between 6 and 8 ppm so as to remove the C-4-H area from this part of the spectrum. The remaining area was normalized to 58 protons per H3/H4 pair. In this way the number of protons corresponding to each resonance peak was estimated for an H3/H4 pair. The accuracy is not great due to the considerable peak overlap.

Essentially four perturbed peaks can be defined in the aromatic spectrum: Ar 1 at 6.65 ppm, Ar 2 at 6.45 ppm, Ar 3 at 6.29 ppm, and Ar X in the region of 6.98 ppm.

It is difficult to assess the area of peak X and figures are thus given only for the other three together with the area of the tyrosine ortho peak at 6.80 ppm see Table I.

If peak X is taken as about seven protons, then a total of about 16 or 28% of the aromatic protons are perturbed. In the folded form of globular proteins a high proportion of the aromatic resonances experience a significant shift, and the relatively low number observed with the H3/H4 histone indicates that the structure is far from being completely globular. The content of aromatic residues is of course low, but histone H5 is a worthwhile comparison in that the aromatic content is comparable, yet the folded form shows a highly perturbed aromatic spectrum (Bradbury et al., 1973).

It has been noted for the H3/H4 complex that the histidine C-2 protons have almost coincident chemical shifts throughout their titrations, showing that the histidines (for H3 at 39 and 113 and H4 at 18 and 75) have very similar p*K*'s. Most globular proteins exhibit a great dispersity in the p*K* of their histidine residues.

Three upfield ring current shifted peaks are observed (see Figure 1) at 0.71, 0.50, and 0.23 ppm (A, B, and C, respectively). While these are also diagnostic of tertiary structure, it was not possible to measure their area with any accuracy, due to their considerable breadth.

Comparison of the spectra of native $(H3/H4)_2$ and denatured (6 M urea) histone shows that a large proportion of the glycine α-CH_2 resonances at 4.0 ppm occur in the spectrum of the native form, Figure 6a(i), at exactly the same chemical shift and line width as in the spectrum of the random coil form, Figure 6a(v). This is an important observation because the glycine α-CH_2 groups are in the backbone of the histone polypeptide chains and it demonstrates that extensive regions of the histone molecules containing glycines are not restricted in the interacted state of the H3/H4 tetramer. There are a total of 48 glycines in the H3/H4 tetramer, of which 28 (59%) are in the very basic N-terminal third of each molecule, and an additional 10 (21%) in the immediate C-terminal ends of the molecules (H4, glycines 94, 99, 101, and 102 and H3 glycine 132). From the behavior of the glycine α-CH_2 resonance it is reasonable to propose that the N-terminal regions of histones H3 and H4 are not involved in complex formation, but are as mobile as the random coil form. The immediate C-terminal ends of H3 and H4 may also be mobile.

Conclusions

The ^{1}H NMR spectra of the native H3/H4 tetramer show it to be far from fully globular, and this conclusion is fully borne out by the high frictional ratio of 1.99 calculated from the sedimentation velocity experiments. The observation of a small number of perturbed resonances does, however, demonstrate the presence of some specific tertiary structure in the complex, which is probably associated in some way with the very considerable amounts of α helix (29 ± 2% by the present estimate). Denaturation by 6 M urea leads to loss of both the secondary and tertiary components of structure, but subsequent dialysis back to water, or even to 10^{-2} M HCl, causes the complex to re-form essentially in its native state. The renatured complex in 50 mM acetate/50 mM bisulfite shows identical spectra to the native complex.

A frictional ratio of 1.99 can be explained in terms either of a highly expanded and hydrated particle or of an asymmetric particle. One possible model would be that only a part of the four polypeptide chains is folded into a central compact and essentially spherical core (presumably the more hydrophobic C-terminal regions), while the remaining residues are randomly coiled and fully flexible in solution. The helical segments are presumably included in the compact regions, and some recent evidence suggests that the helical region of H4 is in the C-terminal rather than the N-terminal portion of the chain (Lewis et al., 1975). The proportion of residues included in the globular core of such a structure must exceed the helicity by a significant factor on the basis of known globular protein folding. If the maximum possible helicity of a globular fold is taken as about 70% (the figure for myoglobin), then with a helicity of 29% the lowest possible "globularity" is about 45%. The overall shape of such a complex would be approximately spherical, and with 55% of the residues free in solution a frictional ratio of 1.99 is quite conceivable. A possible objection to this model is that a globular fold containing nearly 200 residues might be expected to show more perturbed resonances in its NMR spectrum. This objection cannot be quantified since the number of significantly perturbed resonances depends critically on the distribution of aromatic residues in the fold, which remains an unknown factor at present.

A second possible model would be a somewhat asymmetric particle whose structure is based essentially on the helical component. A 29% helicity implies a total helical length of some 200 Å (divided among the four molecules). A side-by-side interaction of helicies would probably not give rise to strong chemical shift perturbations in the NMR spectrum, and such a structure could represent a framework for assembly of a chromatin subunit.

Whichever model of the tertiary and secondary structure is nearer to the truth, the NMR spectra show clearly that certain portions of the chain remain free and unrestricted under all conditions studied. The observation of an intense and narrow resonance from glycine CH_2 groups in the native complex demonstrates that a majority of these residues and, therefore, the backbone in their proximity are unrestricted. Since glycines are concentrated in the N-terminal regions (particularly for H4), it follows that these sections remain free and, therefore, require an additional component to assume their native structure; this is presumably the DNA and/or the other histones H2A and H2B.

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