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THE INTERACTION OF HISTONE H3 WITH HISTONE H4 AND WITH OTHER HISTONES STUDIED BY ^{19}F NUCLEAR MAGNETIC RESONANCE

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SUMMARY

The behaviour, upon variations in ionic strength, pH and temperature of ^{19}F nuclear magnetic resonance signals of the trifluoroacetonylated derivative of histone H3 is compared with those of the H3·H4 complex and of the Hν fraction (an equimolar mixture of H2A, H2B, H3 and H4). The line width of the ^{19}F-labelled histone H3 signals increases with ionic strength or pH, an effect consistent with aggregation of the protein. In the case of H3·H4 complex or Hν the line width decreases at intermediate ionic strengths (0.1–0.25 M NaCl). This effect is interpreted as the consequence of the formation of a well defined structure with ionic strength. At high salt concentrations the line width increases as a consequence of the final rigid quaternary structure or of the formation of higher aggregates.

INTRODUCTION

The discovery of the subunit structure of chromatin has drawn the attention to the conformational problems involved in the formation of histone oligomers. It has been shown that histones H3 and H4 form a complex when they are extracted by gentle procedures [1] and the existence of a tetramer $(H3 \cdot H4)_2$ has been proposed [2, 3]. This complex can be formed in solution from individual fractions under proper conditions [4], and the proximity of these proteins in solution and in chromatin has been shown using cross-linking reagents [5, 6]. Histones H2A and H2B can also form a complex of an 1:1 stoichiometry [5, 7, 8]. The relationship between this oligomer and that formed by H3 and H4 is not yet clear at the present time. Nevertheless the proximity within chromatin of histone H2B and H4 has been shown [9] and the existence of an octamer [10] or a tetramer [11] formed by two or one molecule from each fraction has been proposed.

The conformational properties of histone H3 have been studied by using spectroscopic techniques and taking advantage of the presence of special residues such as cysteine [12, 13] or tyrosine [14, 15]. H3·H4 complexes have been studied in a similar way, and the stoichiometry of the complex has been confirmed [16]. It also

appears that the conformational transition related to aggregation in H3 and H4 is completely inhibited by complex formation [16].

NMR studies [17, 18] on histone H3·H4 complexes point to the existence of a globular core with flexible tails sticking out, where the interactions with the phosphate groups of DNA can be located. Such a model, in fact, might be anticipated even by simple inspection of sequences of histones H3 and H4. In these two histones the basic residues are concentrated in the N-terminal segments of each histone, while helix-forming residues are mainly present in the central and C-terminal parts of the sequence [12, 19, 20]. The two cysteinyl residues of H3 (Cys-96, Cys-110) are located in the region with high potential for structure formation. In this sense, a tertiary structure has been detected in this region [12, 13], which is sensitive to the formation of complexes with H4 [21].

Taking advantage of the high reactivity of the cysteinyl residues [13] a specific labelling approach to the problems of oligomer formation is possible. ^{19}F NMR spectroscopy has been used in this way in a variety of problems, e.g. enzymes and hemoglobin [22]. The advantage of this nucleus is its high sensitivity, especially if Fourier Transform techniques are used. It seems, therefore, a suitable technique to study the formation of the H3·H4 complex, the conformational changes that it induces to H3 and the effect of other histones on this complex.

MATERIALS AND METHODS

Calf thymus histone H3 was prepared following the method of Johns [23] modified by Palau and Daban [13] and histone H4 following the method of Phillips and Johns [24] modified by Climent [25]. The purity of these fractions was determined by electrophoresis [26] and amino acid analysis. Fraction Hν was obtained by extraction with 0.25 M hydrochloric acid from a calf thymus chromatin which was previously depleted from H1 by treatment with 5% perchloric acid. This fraction contains histones H2A, H2B, H3 and H4 in an equimolar proportion as seen by electrophoretic analysis.

Bromotrifluoroacetone (3-bromo-1,1,1-trifluoropropanone) was a product from Penninsular Chem. Res. Inc., 5,5′-dithiobis (2-nitrobenzoic acid) (Nbs_2) was purchased from Aldrich, and all other chemicals from Merck.

Complexes between histones H3 and H4 were made following the procedure of D'Anna and Isenberg [4] without dialysis in order to avoid as much as possible the oxidation of the SH groups of histone H3. Concentration was calculated directly from the weight of the samples and the volume of the solutions. The labelling of histones and complexes was done at 2.5 mM phosphate pH 7.0 by adding 4–6 mol of bromotrifluoroacetone per mol of H3. The reaction mixture was left for 4 h and the pH was controlled during this time and adjusted to 7.0 if necessary. The extent of labelling of SH groups was controlled by means of the Ellman's reaction [13] with Nbs_2 and it was of 70% in histone complexes and 100% in H3 alone. Extensive dialyses against 2.5 mM phosphate/0.1 M NaCl, pH 6.5, were carried out in order to eliminate the excess of bromotrifluoroacetone and, finally, the solution was dialysed against the desired solution. Changes in the ionic strength or in pH of a given solution were obtained, respectively, by adding either solid sodium chloride or 0.1 M HCl.

^{19}F NMR spectra were recorded at 94.1 MHz using an XL-100 Varian spectrometer operating in the Fourier Transform mode. 10% ^2H_2O was added for locking. Sample concentration was less than 10 mg/ml of H3 in all cases. The number of scans was between 2000 and 7000. The measurements were carried out in 12-mm calibrated tubes and using an internal 1 mm capillary tube containing an aqueous solution of trifluoroacetic acid as a reference. Temperature measurements were carried out before and after each run.

Gel filtration chromatography of fraction Hν at high salt concentration was carried out according to A. Ruiz-Carrillo (private communication) in a Bio-Gel P-200 column. Elution buffer was 2 M NaCl/1 mM Tris/2 mM EDTA/0.05% 2-mercaptoethanol/0.1 mM phenylmethylsulphonyl fluoride, pH 7.2, at 4 °C. 1 ml samples were collected and precipitated with trichloroacetic acid, turbidity was measured and taken as a measure of protein concentration. Slab gel electrophoresis was carried out in 13–18% gradient polyacrylamide according to Laemmli [27].

RESULTS AND DISCUSSION

^{19}F-labelled histone H3

Fig. 1 shows the ^{19}F resonances of the CF_3 groups linked to histone H3 under different conditions of temperature, ionic strength and pH, and under the effect of guanidinium chloride. A reasonably sharp single resonance band centered at 812 Hz upfield from trifluoroacetic acid is observed at 30 °C in a solvent medium (water, pH 3.1) where the histone molecules are supposedly in a random coil form [20]. The

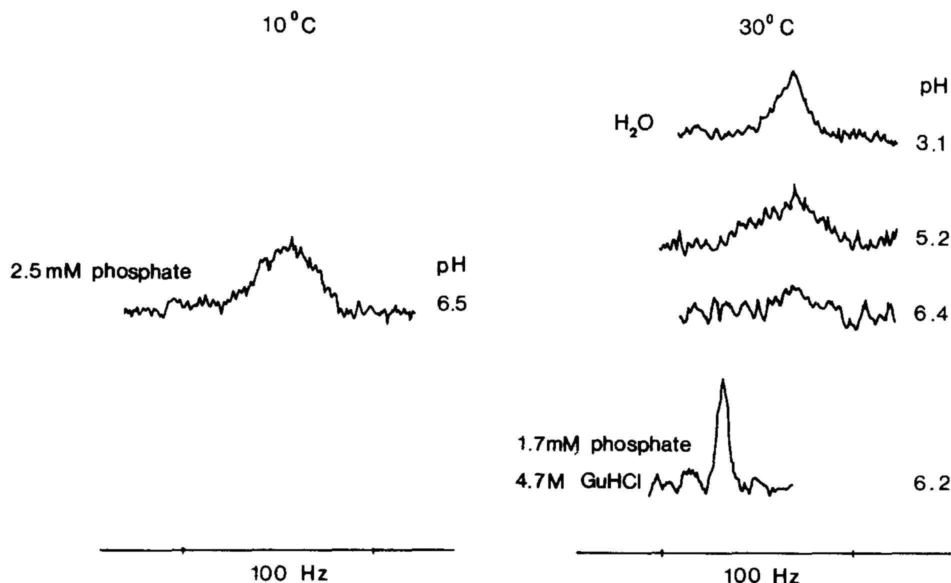


Fig. 1. ^{19}F NMR spectrum (at 94.1 MHz) of histone H3 labelled with bromotrifluoropropanone in 2.5 mM phosphate at 30 °C and at 10 °C (4–10 mg/ml). The chemical shift of CF_3 resonances is peaked around 812 Hz at 30 °C (around 835 Hz at 10 °C) upfield from trifluoroacetic acid. Addition of 4.7 guanidinium chloride induces a low-field chemical shift of 30 Hz (after correction of the shift produced on lock signal).

shape of the band is asymmetric in most cases as it would be for two partially overlapping peaks. This may be an indication of the existence of two slightly different environments for the labels attached to the two cysteine residues of the molecule. Nevertheless the asymmetry is not large enough to allow any study of differential effects.

An increase in the pH of the solution within the acidic range induces a broadening of the resonance, to such an extent that at pH 6.4 it is barely visible above noise level. Such a behaviour is certainly consistent with the accepted view for arginine-rich histones of pH-induced or salt-induced formation of large aggregates. If the temperature is lowered to 10 °C a broad band is visible at pH 6.5, an effect which may indicate that aggregation in histone H3 decreases with the lowering of temperature probably due to the decrease in strength of the hydrophobic interactions. If the ionic strength of the sample at low temperature is increased in 0.1 M NaCl the signal disappears completely, thus indicating a process of self-aggregation.

Guanidinium chloride is a strong denaturating agent for proteins [28]. If a sample of ^{19}F-labelled H3 is dissolved in 4.7 M guanidinium chloride a single sharp and symmetric resonance is observed. This indicates that only cysteinyl residues are labelled because the signal is unique, and it favours the hypothesis of aggregation as the cause for the broadening observed in the experiments described above. It must be pointed out that the peak observed in the guanidinium chloride experiment is also shifted 30 Hz to low field. This shift can be due to a strong interaction of the CF_3-CO- group with the guanidinium ion. A similar, although stronger, effect was observed in a labelled model compound, i.e. ^{19}F-labelled glutathione. In this case the resonance, which in the absence of guanidinium chloride is observed at 40 Hz to high field with respect to the labelled histone, is shifted by 54 Hz to low field in the presence of guanidinium chloride. Assuming that guanidinium chloride has the same effect on the model and the random coil protein, producing a shift of 54 Hz to low field, either the CF_3-CO- groups in the protein are at least partially protected from the interaction with the guanidinium ion, or the formation of the structure in the protein produces a chemical shift of 24 Hz to high field.

^{19}F-labelled H3·H4 complexes and Hv fraction

For H3·H4 complex dissolved in 2.5 mM phosphate, pH 6.5 at 10 °C, the ^{19}F resonance of the CF_3-CO- group attached to it is centered at the same position (835 Hz upfield from trifluoroacetic acid) as in the case of ^{19}F-labelled histone H3 under the same conditions, and it has a line width of about 24 Hz (Fig. 2). A remarkable fact shown in Fig. 2 is that an increase of the ionic strength has an opposite effect in comparison to that found for histone H3 at intermediate (0.1 M) NaCl concentrations. The resonance peak reaches a minimum line width at salt concentrations between 0.05 and 0.5 M NaCl. With the increase of salt the signal undergoes a gradual shift to low field and at 0.75 M NaCl it reaches 26 Hz from the position in the absence of salt. pH and temperature produce an increased broadening of the signal. At 0.75 M NaCl the variation of pH to 3.2 causes a complete disappearing of the peak, and if the temperature is changed from 10 to 30 °C in 2.5 mM phosphate pH 6.5 the line width increases from 24 to 42 Hz. Complex H3·H4 dissolved in 4.7 M guanidinium chloride shows a signal which is identical in position and shape to that found for H3 under similar conditions.

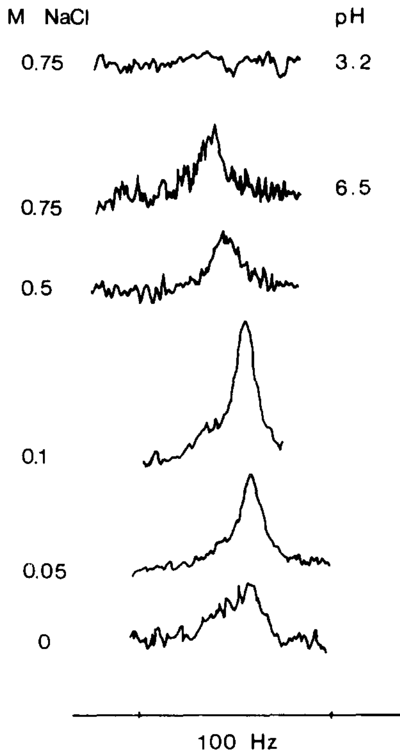
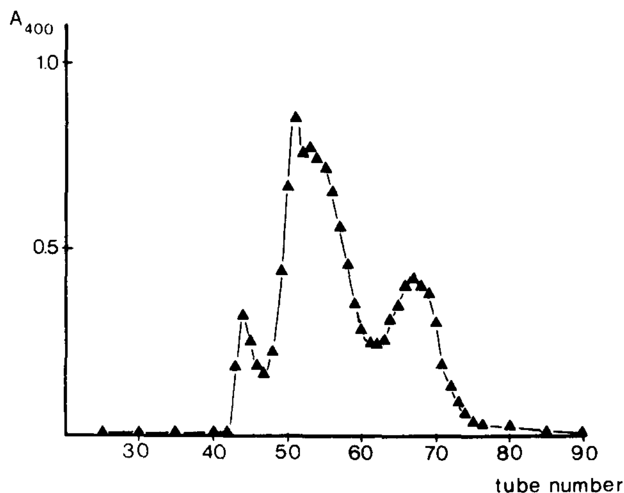


Fig. 2. ^{19}F NMR spectrum of ^{19}F-labelled H3·H4 complex in 2.5 mM phosphate at 10 °C. Effect of NaCl concentration and pH.

Effects in chemical shift and line width similar to those found for H3·H4 are also observed for the fraction Hν. This fraction has a pattern of oligomer distribution in 2 M NaCl (Fig. 3) very similar to that described by Weintraub et al. [11] for whole histones extracted by gentle procedures. This result indicates that as compared with non-acid extractions the interactions between histones in Hν are preserved during the process of extraction or can be reformed in solution. On Fig. 4 the effect of NaCl concentration on the resonances of ^{19}F-labelled Hν is shown. The main difference between these effects and those described for H3·H4 are that at high salt concentration the line broadening is larger and the effect of pH is less pronounced.

As it has been presented in the preceding paragraphs both ^{19}F-labelled H3·H4 and Hν undergo a narrowing upon increasing ionic strength until intermediate values (0.1–0.25 M NaCl). This effect is the reverse of that observed for H3. It has been shown that formation of the H3·H4 complex prevents aggregation [16], thus the main reason for line broadening in histone H3 is eliminated. But to understand why a decrease of line width is observed the contribution of exchange broadening has to be taken into account. Indeed, it has been shown that at acid pH some structure is formed in the H3·H4 complex with the addition of NaCl [18]. The narrowing observed in the ^{19}F resonances may then be attributed to the adoption of a stable tertiary structure in the environment of the Cys residues. The fact that the same



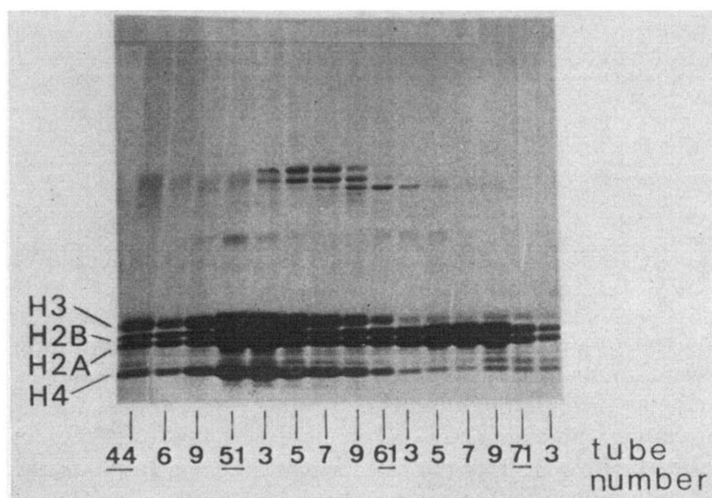


Fig. 3. Chromatography of the Hν fraction through a Bio-Gel P-200 column in 2 M NaCl/1 mM Tris/2 mM EDTA/0.05% 2-mercaptoethanol/0.1 mM phenylmethylsulphonyl fluoride, pH 7.2 at 4 °C. Turbidity was measured at 400 nm in 18% trichloroacetic acid.

behaviour is observed for Hν indicates that in this conditions the structure of the Cys-containing regions of the H3·H4 complex is probably independent of the presence of the other two fractions.

It has been presented above that at concentrations higher than 0.25 M NaCl the line width increases for both ^{19}F-labelled H3·H4 and Hν, the effect being larger for the last one. This can be a consequence of the formation of the final rigid quaternary structure (indeed Weintraub et al. [11] proposed that H2A, H2B, H3 and H4 complex exists only at high ionic strength) or an effect of aggregation [18] of the complex at those high concentrations of NaCl and of protein. The absence of a large increase in line broadening as that observed by Moss et al. [18] for H3·H4 and

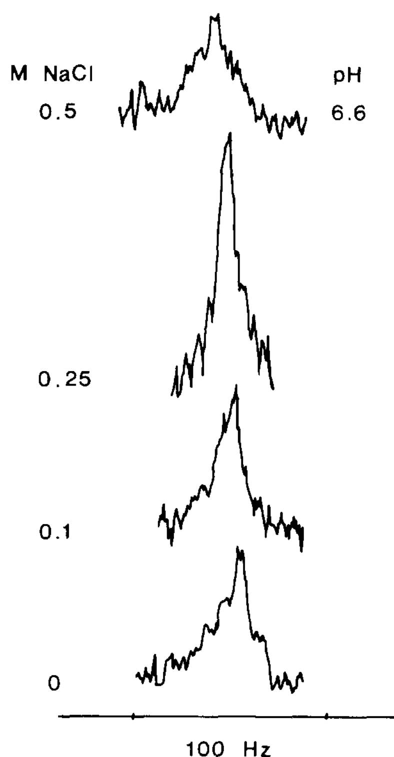


Fig. 4. ^{19}F NMR spectrum of ^{19}F-labelled Hν fraction in 2.5 mM phosphate at 10 °C, pH 6.6. Effect of NaCl concentration.

attributed to aggregation can be due to the lower temperatures and concentration used in our experiments and points to the first hypothesis. In this sense the presence of other histone fractions may increase the molecular weight of the complex or the ability to form larger and more rigid aggregates.

In both H3·H4 and Hν a lowering of the pH at high salt concentration produces a large broadening. In conditions of acid pH it is plausible to think that repulsions between the proteins that form the oligomers take place; the high salt concentrations would then favour the self-aggregation of histones.

The chemical shift varies to low field with ionic strength in H3·H4 and Hν. These changes follow those in line width that can be interpreted as due to changes in tertiary and quaternary structure. The variation in chemical shift indicates a variation in the environment of the CF_3 group attached to histone H3. The sense of the shift to low field agrees with the fact that the crevices where cysteines are located are 0.8 nm deep as measured by ESR of spin labels (Padrós, E. and Palau, J., manuscript in preparation) and the trifluoroacetonyl groups are therefore near to the surface of histone H3. In this situation the CF_3 group may have polar groups from the same or another molecule nearer upon formation of tertiary and quaternary structure.

It can be concluded that the ionic strength is able to stabilize a tertiary structure of histone H3 in the H3·H4 complex in presence or absence of other histones, at least in the environment where the cysteine residues are located. This form is in

equilibrium with a more rigid quaternary structure or higher aggregates that can be formed by increasing ionic strength. Higher temperatures (30 °C) produce more aggregation, whereas the decreasing of pH to acid values is able to destabilize the specific complex thus allowing at high ionic strength the self aggregation of histones.

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