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Studies on the Role and Mode of Operation of the Very-Lysine-Rich Histones in Eukaryote Chromatin

The Conformation of φ1 Histones from Marine Invertebrate Sperm

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Proton magnetic resonance, circular dichroism and infrared spectroscopy are used to investigate the secondary and tertiary structures of three very lysine-rich histones from marine invertebrate sperm. At high ionic strength both *Arbacia lixula* and *Holothuria tubulosa* histone φ1 are observed to contain 25–30% α-helix, no β-structure and to form specific folded structures. Both φ1 proton magnetic resonance spectra have perturbed methyl resonances at chemical shifts close to those observed for calf thymus H1, suggesting analogies in tertiary structure. *Mytilus edulis* histone φ1 however, shows no spectroscopic evidence of secondary and tertiary structure on salt addition.

The formation of specific folded structures in calf thymus histone H1 induced by increase of ionic strength or of pH has been demonstrated in the first paper of this series [1]. It appears that in histone H1 a highly ordered structure exists, which involves at least the central and less hydrophilic region of the molecule. This conclusion follows from the appearance of signals in the high-field part of the nuclear magnetic resonance (NMR) spectra of the protein, which are shifted by the ring-current effects of aromatic side chains. Furthermore the circular dichroism (CD) spectrum of H1 demonstrated the presence of some α-helix in the folded state.

In the present work we have extended our conformational studies to a group of sperm histones from marine invertebrates (coded as φ1 histones) which have been extracted from the species *Arbacia lixula* (sea urchin), *Holothuria tubulosa* (sea cucumber) and *Mytilus edulis* (sea mussel). These basic proteins can be considered analogous to histone H1 from somatic tissues of vertebrates in the sense that they have been prepared in the same way as histone H1 by the methods of Johns [2], they are electrophoretically homogeneous [3,4] and, with the exception of *M. edulis* histone φ1, they resemble calf thymus histone H1 in amino acid composition (see Table 1). The most striking features of the composition of these histones are the great variability in arginine content, the small number of amino acids present in histone φ1 of *M. edulis* and the considerable variation in aromatic residues and in histidine. Despite these variations they are clearly closely related to calf thymus H1 histone.

As earlier stated [3] the high basicity of these histones could be related to the synthetic inactivity of the sperm nucleus as a whole. This also seems to be the case in different types of sperm [6] and in avian erythrocytes [7,8] which possess the additional histone H5, rich in lysine and in arginine.

Our aim in this paper is to investigate structural homologies and differences within the φ1 histones and to compare them with calf thymus histone H1. Such data are important to define which aspects of the secondary and tertiary structure of these histones are relevant for the preservation of any specific (though at present unknown) functionality of the H1 and φ1 molecules. In addition conformational studies on sperm histones may give a clue as to the state of the genome in these type of cells.

Abbreviations. NMR, nuclear magnetic resonance; CD, circular dichroism.

EXPERIMENTAL PROCEDURE

Nuclear Magnetic Resonance Spectra

Spectra at 270 MHz were run on a Bruker WH-270 spectrometer. Solutions were made up directly in ^2H_2O (99.8%). Standard 5-mm tubes were used, and

Table 1. *Amino acid composition of several very lysine-rich histones and histone peptides*
Results are expressed as a percentage of the total residues for each histone

Amino acid	Amount in histone					
	calf thymus H1	*A. lixula* φ1			*H. tubulosa* φ1	*M. edulis* φ1
		total	C-terminal	N-terminal		
Lysine	27.4	27.4	31.9	19.9	33.5	22.2
Histidine	–	1.2	–	–	–	–
Arginine	1.8	11.2	10.7	12.6	3.9	29.4
Aspartate/ asparagine	2.2	2.2	1.9	2.1	3.4	0.2
Threonine	5.6	2.5	2.9	1.4	4.1	3.8
Serine	7.2	6.7	5.3	12.0	3.2	17.7
Glutamate/ glutamine	3.8	2.0	0.8	3.9	5.3	0.2
Proline	9.2	9.0	7.9	14.7	5.7	5.5
Glycine	7.0	4.6	4.3	5.3	4.5	5.7
Alanine	24.3	23.6	28.0	19.0	25.1	13.8
Valine	5.0	2.8	2.7	2.5	4.7	1.0
Methionine	–	1.2	–	1.5	0.2	–
Isoleucine	1.0	2.8	1.7	3.8	2.8	0.1
Leucine	4.5	1.4	1.3	0.9	2.7	0.2
Tyrosine	0.5	0.8	–	–	–	0.1
Phenylalanine	0.5	0.4	0.5	0.1	0.9	0.1

spectra were obtained by transforming the sum of between 10000 and 40000 free induction decays accumulated over periods from 2.5 h to 10 h. The long-term accumulation was carried out using a double-precision accumulation programme (G. E. Chapman, unpublished) and convolution difference methods [9] were used for the resolution enhancement in Fig. 1. Shifts were measured relative to sodium 2,2-dimethyl-2-silapentane sulfonate.

Circular Dichroism Spectra

CD spectra were recorded on a Cary 61 spectropolarimeter using a Spectrosil cell of path-length 0.013 cm. The results are expressed as the mean residue ellipticity $[\theta]_\lambda$ (deg. cm^2 dmol^{-1}) and were not corrected for the refractive index of the solvent. The mean residue weight was calculated from the amino acid composition of each protein. Helix content was calculated according to a modification of the method of Chen *et al.* [10] that is discussed elsewhere [11] and which consists essentially in using the CD spectrum of certain histone fragments as the random coil standard.

Infrared Spectra

Infrared spectra were recorded on a double-beam Grubb Parsons Spectromaster at room temperature with barium fluoride cells and 0.075-mm spacers. A variable path-length cell containing ^2H_2O was used in the background beam.

It is important to point out that all three physical measurements were made at the same protein concentration ($\approx$ 1 mM or 20 mg/ml) so as to avoid any concentration dependence of conformation when comparing the results from different techniques.

Preparation of φ1 Histones

The sperm was extracted and purified as described elsewhere [12]. The histone φ1 from *A. lixula* sperm was prepared essentially as in Palau *et al.* [3]. The purified sperm was extracted with 5% $HClO_4$ (5 ml/g of wet sperm) by homogenising in a high-speed blender (30 s at maximum speed) and then by gently stirring for 1 h. The homogenate was centrifuged for 30 min, at 2500 × *g* and the supernatant was made 0.25 N in HCl. The solution was stirred and acetone was added until the formation of a precipitate. The suspension was centrifuged and the sediment was washed with acetone three times and dried under vacuum. Electrophoretic analyses on polyacrylamide gels [13] and amino acid composition (see Table 1) demonstrated the high purity of the material.

The preparation of φ1 histones from *H. tubulosa* and from *M. edulis* sperm was carried out in a similar way to that described above for *A. lixula*.

Cleavage Using N-*Bromosuccinimide, of Histone φ1 from* A. lixula *Sperm*

The procedure of hydrolysis was, essentially, that described in a preceding paper [1] and uncleaved histone φ1 was not observed as monitored by electro-

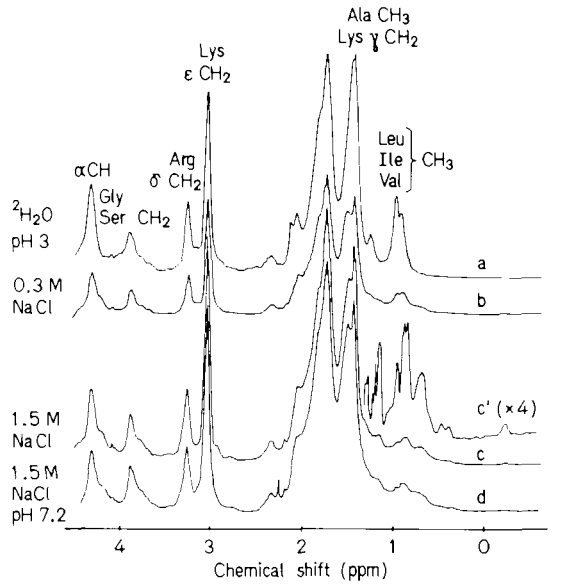

Fig. 1. *High-field 270-MHz NMR spectra of histone φ1 from* Arbacia lixula *sperm at 20 mg/ml.* (a) ^2H_2O, pH 3, (b) 0.3 M NaCl, (c) 1.5 M NaCl, (c') the same as (c) after resolution enhancement by convolution difference and multiplied 4 times, (d) 1.5 M NaCl, pH 7.2

phoresis. In order to separate the cleaved halves the hydrolysis product was made 0.25 N in HCl and acetone was added to the solution until precipitation occurred (at 1 vol. of acetone). The precipitate that formed was collected (C-terminal fraction) and more acetone (0.3 vol.) was added to the supernatant. The new precipitate that appeared (a mixture of C-terminal and N-terminal fractions) was separated and a further 0.6 vol. of acetone was added to the supernatant to precipitate the N-terminal fragment. The precipitates were washed three times with acetone and dried under vacuum. The total yield was 50% of the starting material. Polyacrylamide gel electrophoresis [13] indicated the presence of three bands for the N-terminal fraction and two slower bands for the C-terminal peptide. This multiplicity could be accounted for by the presence of two tyrosine residues located rather near to each other in the N-terminal part of the chain. It appears that the yield of the cleavage with *N*-bromosuccinimide is not 100%, and therefore two N-terminal peptides and an intermediate one of low molecular weight, and two large C-terminal peptides should be expected. The amino acid analyses are given in Table 1.

RESULTS AND DISCUSSION

Histone φ1 from A. lixula *Sperm and Its Fragments*

The NMR spectra of histone φ1 from *A. lixula* at different concentrations of NaCl were studied (Fig. 1 and 2). The spectrum in pure ^2H_2O corresponds closely to that expected for a random coil state. The addition of salt produces changes at both high field and low field from ^2H_2O. At high field the main changes appear in the peak located at about 0.9 ppm from sodium 2,2-dimethyl-2-silapentane sulfonate which corresponds to the methyl groups of valine, isoleucine and leucine. In fact, well over half of these CH_3 groups are significantly perturbed on salt addition. The decrease in apparent area of this peak with the increase of salt concentration is accompanied by an increase in the areas of signals appearing at high and low field from it. This is very apparent in the convolution difference spectrum C′ of Fig. 1. This effect, observed also in histone H1, is interpreted as the result of the magnetic anisotropy of aromatic rings in close proximity to valine, isoleucine or leucine residues. The area of the peak at −0.2 ppm at high salt concentration and neutral pH, measured with respect to the area of the doublet tyrosine peak centered at 6.8 ppm (four protons), corresponds closely to 3 protons, showing that it is indeed a methyl resonance shifted from its position in ^2H_2O and pH 3.

Spectra of histone φ1 obtained earlier at 220 MHz [14] did not show evidence of ring-current-shifted peaks since the lower signal-to-noise ratio then obtained was insufficient to observe single CH_3 resonances. The present observation of perturbed CH_3 peaks indicates that the protein has a specific and well-defined tertiary fold in at least a part of its polypeptide chain. As in the case of calf thymus histone H1, ring current effects were also observed in the absence of salt as the pH was raised from 3 to 7. In addition, it is worth noting that salt addition did not lead to line-broadening in the φ1 spectrum: this is a similarity to calf thymus H1 and it is in contrast to the salt-induced line-broadening in the spectra of H2A, H2B, H3 and H4 that it is caused by histone association. Thus histone φ1 shows no evidence of aggregation under the conditions studied.

The amino acid composition of histone φ1 from *A. lixula* shows some differences when compared with that of calf thymus histone H1. The most striking are the large amount of arginine in φ1, the presence of two histidines and probably two tyrosines. Preliminary sequence results on this protein (J. Palau and O. Cabré, unpublished) show that the supplementary arginine is found largely at both ends of the molecule where most of the lysyl residues are located, leaving the central region with less hydrophilic character similar to that of calf thymus H1.

In both proteins three peaks are apparent at very high field, one at about −0.2 ppm and the others as a pair at about 0.4 and 0.5 ppm. The similarity of the positions of these peaks for the two molecules indicates that the tertiary structure of the φ1 and H1 molecules could indeed be homologous.

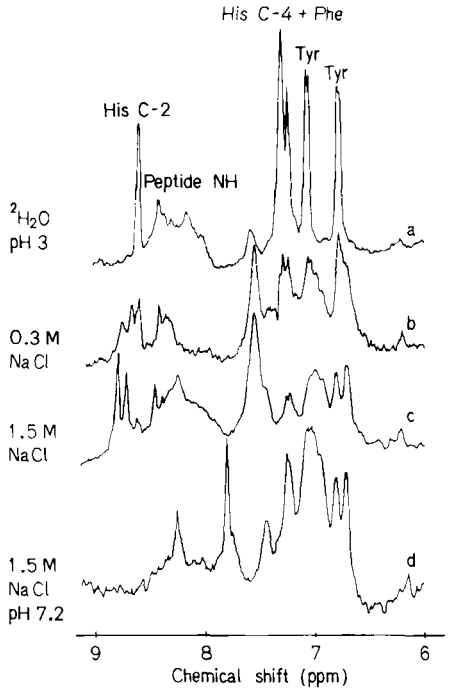

Fig. 2. *Low-field 270-MHz NMR spectra of histone φ1 from* Arbacia lixula *sperm at 20 mg/ml.* (a) ^2H_2O, pH 3, (b) 0.3 M NaCl, (c) 1.5 M NaCl, (d) 1.5 M NaCl, pH 7.2

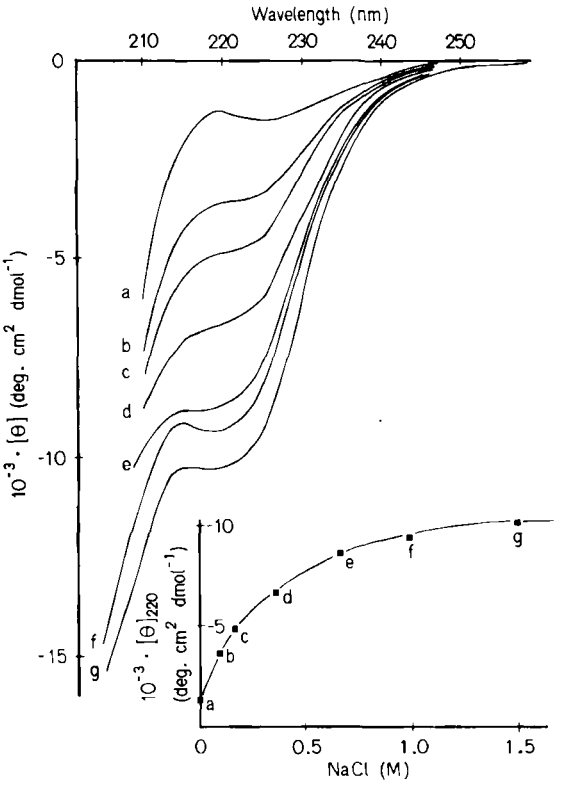

Fig. 3. *Circular dichroism spectra of* Arbacia lixula *histone φ1 at different ionic strengths at 20 mg/ml.* (a) H_2O, pH 3, (b) 0.1 M NaCl, (c) 0.16 M NaCl, (d) 0.36 M NaCl, (e) 0.66 M NaCl, (f) 1.0 M NaCl, (g) 1.5 M NaCl

The low-field spectrum of histone φ1 from *A. lixula* sperm is shown in (Fig. 2). In ^2H_2O at pH 3 the relative peak areas correspond closely to two histidines, two tyrosines and one phenylalanine, with both histidines and both tyrosines being equivalent. This is the spectrum expected for a highly disordered conformation. Resonance between 8.0 and 8.5 ppm is due to slowly exchanging peptide NH that could result from a small degree of residual order under these conditions. On salt addition there is clear evidence of chain folding and consequent perturbation of several resonances. For example, two new histidine C-2 peaks appear at 8.7 and 8.8 ppm whilst the 8.6-ppm peak gradually disappears. Both histidines thus show some perturbation and are presumably included in the tertiary fold. The co-existence of histidine C-2 peaks characteristic of the disordered and of the folded forms (*e.g.* in 0.3 M NaCl) demonstrates that the exchange rate between the two forms is slow ($< 150\ s^{-1}$). Similar behaviour is noted for the two tyrosine residues: the upfield peak at 6.80 ppm splits on salt addition into two peaks that correspond to the two different tyrosine residues. This peak corresponds to the 2 protons *ortho* to $-OH$ and these protons are typically equivalent magnetically even in globular proteins. At high salt concentration one peak is perturbed to 6.70 ppm whilst the other remains at 6.80 ppm: at least one tyrosine is therefore included in the folded portion of the chain. Although a complete assignment of phenylalanine resonance in the folded form is somewhat uncertain, it is more complex than in the disordered form and it follows that this single residue is also included in the folded region. This complex behaviour of the aromatic spectrum on salt addition occurs also for the other lysine-rich proteins H1 [1] and H5 [15] and for them also such changes have been attributed to protein folding. The absence of sufficient sequence data for histone φ1 prevents detailed conclusions being drawn on the exact portion of the chain included in the fold.

The infrared and CD spectra allow an examination of the secondary structure of the protein. Infrared spectra of *A. lixula* histone φ1 in the 6-μm region were obtained in pure ^2H_2O (25 mg/ml) and in ^2H_2O/1.5 M NaCl (pH 3). The amide I band remained essentially unchanged on salt addition and in particular there was no new band at $\approx 1610\ cm^{-1}$ that would indicate the formation of β structure (in amounts greater than 5%). The absence of β structure was confirmed by the CD spectra, Fig. 3, (no evidence of any negative band at $\approx$ 217 nm that appears normally to remove the dip between the two bands at 222 and 205 nm characteristic of α-helix). The CD spectra also demonstrate the formation of α-helix. As the ionic strength of the solution increases there is a sharp rise in the negative ellipticity at 222 nm to a value of about $-10000°$ which represents 25–30%

α-helix. This value is substantially greater than that found for calf thymus H1 [1]. The increase in helicity (see the inset to Fig. 3) occurs simultaneously with the formation of tertiary structure when the latter is monitored by the decrease in the apparent intensity of the 0.93-ppm methyl peak in the NMR spectra or by the changes in the histidine C-2 peaks. For example in 0.3 M NaCl the transition to the form involving both secondary and tertiary structure is about half complete. An equilibrium exists therefore between fully structured and highly disordered chains. Such a situation is typical of the folding of globular proteins, although it is possible that with φ1 and H1 not all of the histone chain becomes included in the fold.

The two fractions of histone φ1 from *A. lixula* sperm obtained by cleavage with *N*-bromosuccinimide were examined. From their amino acid analyses (see Table 1) and by comparison with those for the corresponding fragments of calf thymus histone H1 the two fractions were recognised as N-terminal and C-terminal fragments. Their NMR spectra at increasing salt concentration show no major changes indicating that the necessary requirements within the sequence for the formation of tertiary structure are lost in these peptides. Furthermore, a mixture of both fractions showed no evidence of recovery of the structure. The CD spectra of the C-terminal fraction (Fig. 4) show the presence of a certain amount of α-helix at high salt molarities. When the position of the tyrosine residues has been established this information can be used to help decide the portion of the chain involved in structure formation as has been done for H1 [1].

Histone φ1 from H. tubulosa *Sperm*

The amino acid composition of histone φ1 from *H. tubulosa* shows some differences when compared with the standard H1 histones or with histone φ1 from *A. lixula*. The most interesting features are the presence of a proportion of arginine lower than in the other φ1 histones studied in this paper, but similar to that in H1 and the presence of only one aromatic residue (a single phenylalanine). Ring-current shift effects with increase of salt concentration were also observed in the NMR spectrum of *H. tubulosa* φ1 (Fig. 5). The most clearly detected one is a single methyl peak at −0.13 ppm. As phenylalanine is the only aromatic residue in this protein it must be the cause of this shifted peak. In terms of structural homologies, it is interesting that both *A. lixula* φ1 and calf thymus H1 also show a peak shifted to about −0.2 ppm. It could therefore be that a particular phenylalanine to methyl contact is preserved in all three proteins and is critical to folding. A peak displacement of 1 ppm corresponds to a maximum distance between the aromatic ring and the methyl group involved in the interaction of approximately 0.3−0.4 nm [16].

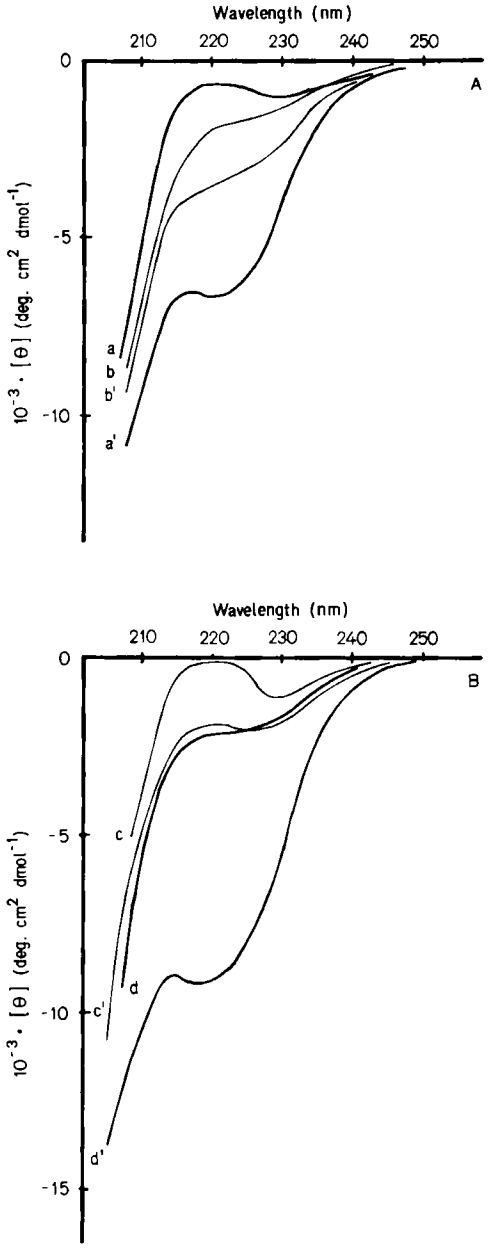

Fig. 4. *Circular dichroism spectra of different histones and histone fragments.* (A) Histone φ1 *N*-bromosuccinimide peptides from *Arbacia lixula:* (a) C-terminal and (b) N-terminal, in H_2O, pH 3; (a') and (b') the same in 1.6 M NaCl, pH 3. (B) φ1 histones from: (c) *Mytilus edulis* and (d) *Holothuria tubulosa*, in H_2O, pH 3. (c') and (d') the same in 1.6 M NaCl, pH 3

The infrared spectra of *H. tubulosa* φ1 in pure ^2H_2O and in solutions at 1.5 M NaCl show no evidence of β-structure as with *A. lixula* φ1. The circular dichroism spectra (Fig. 4) demonstrate the formation of secondary structure with increase of salt molarity. The final state in 1.5 M NaCl shows an ellipticity corresponding to 25−30% α-helix. The capability of this protein for secondary structure formation is thus very similar to that of *A. lixula* φ1. Thus although *H. tubulosa* φ1 has an amino acid composition more

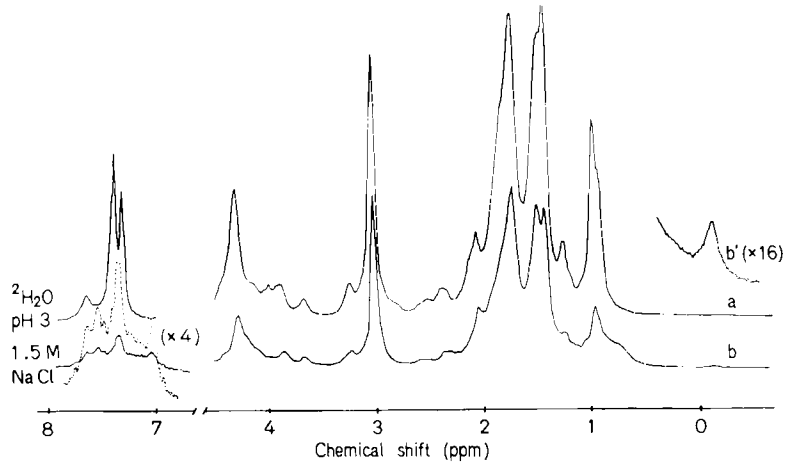

Fig. 5. *270-MHz NMR spectra of histone φ1 from* Holothuria tubulosa *at 20 mg/ml.* (a) ^2H_2O, pH 3, (b) 1.5 M NaCl. (b') is an expansion (×16) of spectrum (b). The broken line is also an expansion of the low-field part of spectrum (b) (×4)

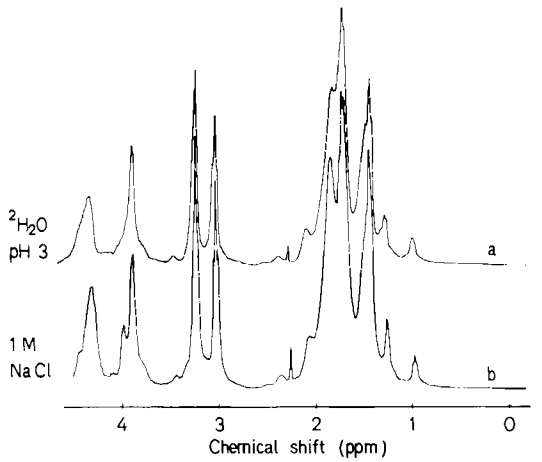

Fig. 6. *High-field 270-NMR spectra of histone φ1 from* Mytilus edulis *sperm at 20 mg/ml.* (a) ^2H_2O, pH 3, (b) 1.0 M NaCl

akin to calf thymus H1 than to *A. lixula* φ1, its secondary structure content is closer to that of the *A. lixula* φ1.

Histone φ1 from M. edulis *Sperm*

The general character of this histone is very different from that of the other two φ1 proteins: in particular its amino acid composition is very simple and corresponds to a protein with a character intermediate between histones and protamines [4].

The NMR spectrum in pure ^2H_2O at low pH (Fig. 6) is that to be expected for a protein in a disordered conformation. Increase in salt molarity does not produce any effect in the NMR spectrum that could be related to a conformational change although the absence of aromatic residues precludes the possibility of ring-current-shifted peaks. The circular dichroism spectrum (Fig. 4) shows an almost negligible change in the molar ellipticity in the region of 220 nm on salt addition indicating that little or no α-helix or β-structure forms. A small negative band at 229 nm is observed in this CD spectrum but its origin is uncertain. The infrared spectrum of *M. edulis* φ1 in ^2H_2O/NaCl solutions up to 1.5 M confirms the absence of β-structure. It can be concluded that histone φ1 from *M. edulis* sperm does not undergo any detectable tertiary folding of the type found for calf thymus H1 and for φ1 histones from *A. lixula* and *H. tubulosa*. The formation of a very limited amount of secondary structure is however possible.

Conclusions

NMR and CD studies show that both secondary and tertiary structure is induced in at least part of the *A. lixula* histone φ1 molecule by increasing ionic strength. No simultaneous aggregation was observed of the type noted for histones other than the very lysine-rich ones. Cleaved fragments of *A. lixula* histone φ1 showed no NMR evidence of folding, indicating the necessity of the presence of complementary parts within the molecule for folding. The overall conformational behaviour was very similar to calf thymus H1 (despite a considerably greater α-helix content in φ1). The pattern of ring-current-shifted peaks in *A. lixula* φ1 is similar to that of H1 suggesting that homologies exist between the tertiary structures of *A. lixula* φ1 and calf thymus H1.

H. tubulosa histone φ1 shows a conformational behaviour very similar to that of *A. lixula* φ1. In particular a ring-current-shifted peak at −0.13 ppm (that also appears in H1 and *A. lixula* φ1) can be attributed to the single phenylalanine residue in the protein. Since histones H1, *A. lixula* φ1 and *H. tubulosa* φ1 all contain a single phenylalanine which is included in the tertiary structure, and which gives rise

to a strongly perturbed methyl resonance, this residue may be important for the folding of all these proteins.

M. edulis histone $\varphi 1$ does not show any detectable secondary or tertiary folding on salt addition. Conformationally it appears to resemble a protamine more than a histone.

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