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On the possibility that H1 histone interaction with DNA occurs through phosphates connecting lysine and arginine side chain groups

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Gel filtration and velocity of sedimentation analyses on native and on lysine- and arginine-modified forms of the annelid worm *Chaetopterus variopedatus* sperm H1 histone indicate that anion-mediated lysine–arginine interactions play a relevant role in the stabilization of the oligomeric states of the molecule. CD spectroscopy shows that phosphate anions are at least an order of magnitude more efficient than chloride as negatively charged groups connecting H1 lysines and arginines. Acetylation of lysines, although not altering grossly the H1 properties, causes a tenfold decrease of the structuring efficiency of phosphates. This suggests that DNA phosphates may be sandwiched between lysine and arginine groups of H1 histone when this molecule binds to chromatin, constituting a relevant parameter for the reciprocal stabilization of the protein and of the chromatin higher order structures.

Keywords: sperm H1 histone, ionic interaction, *Chaetopterus variopedatus*, protein structure, lysine, arginine

INTRODUCTION

Almost all nucleosomes in the chromatin of eukaryotes contain one molecule of the linker histone H1. This type of histone is present as a complex family of related proteins with distinct species- tissue- and developmental-specificity (Khochbin, 2001). H1 histones present a tripartite structure consisting of a central globular domain flanked by highly basic N- and C-terminal tail-like domains that are largely unstructured in solution (Hartman *et al.*, 1977) but acquire a substantial amount of secondary structure upon interaction with DNA (Vila *et al.*, 2001a; 2001b). While the globular domain binds to nucleosomal DNA, the N- and C-terminal domains are regions considered to be involved in chromatin condensation through binding to the negatively charged phosphate groups of the internucleosome linker DNA (Lu & Hansen, 2003; 2004; Hendzel *et al.*, 2004). Interactions between H1 molecules bound to adjacent nucleosomes are considered to stabilize the 30 nm fiber typical of the condensed chromatin structure (Zlatanova & van Holde, 1996; Ramakrishnan, 1997). To date, attempts to determine whether linker histones specifically self-interact in the absence of DNA or chromatin have led to different results from different research groups (Thomas *et al.*, 1992; Draves *et al.*, 1992; Maman *et al.*, 1994). A number of initial studies have indicated that H1 histones and their globular domains have a tendency to self-associate (Russo *et al.*, 1983; Maman *et al.*, 1994) especially when they are bound to DNA (Thomas *et al.*,1992; Draves *et al.*, 1992). We have already reported on the occurrence of anion-mediated arginine-lysine interactions in the sperm protamine of the marine annelid worm *Chaetopterus variopedatus* and confirmed this type of unusual bond in model polyarginine and polylysine molecules (Piscopo *et al.*, 1993). We extend now the investigation on that type of interaction to the formation of higher order aggregates of *C. variopedatus* H1 histone and analyse the structuring capacity of different anions. To this aim we performed specific chemical modifications of lysines or arginines of H1 histone and analyzed

the effects of each of these modifications on the ability of the molecule to form aggregated structures. We report here the results of studies that provide further support to the existence of anion-mediated lysine–arginine interactions and open the possibility that DNA phosphates may act as connecting anions between those residues leading to the reciprocal stabilization of both H1 histone organization and higher order chromatin structures.

MATERIALS AND METHODS

Materials. Chemical reagents and molecular mass marker proteins were obtained from Sigma (USA). Gel filtration media were purchased from Pharmacia (Sweden).

Preparation of H1 histones. *C. variopedatus* sperm H1 histone was purified as reported (De Petrocellis *et al*., 1983). The purity of the protein was analysed by slab gel electrophoresis on 18% polyacrylamide/sodium dodecyl sulfate as described by De Petrocellis *et al.* (1980). The protein concentration was determined on the basis of the known content of two tyrosines per *C. variopedatus* sperm H1 molecule (De Petrocellis *et al.*, 1983) using ε_{276} = 1340 cm^{-1} (mol tyrosine)$^{-1}$ l^{-1} (Giancotti *et al.*, 1981).

Amino-acid side chain modifications. Carbamylation of lysine ε-amino groups of H1 histone was performed by overnight reaction of 1 mg protein in 0.1 mL of 6 M urea, at 30°C, with 1.5 M potassium cyanate. The mixture was then diluted with water to 1 mL and extensively dialyzed against water to remove excess reagent. Succinylated and acetylated derivatives were obtained by dissolving 1 mg of histone in 0.1 mL of water and overlaying the solution on 1 mL of (1:2, v/v) chloroform/dioxane mixture containing freshly dissolved specific anhydride (50 mg/mL and 25 mg/mL for acetic and succinic anhydride, respectively). The reaction, performed for 16 h at room temperature, occurs through the interface between the two solutions. The aqueous phase was then diluted with water to 1 mL and extensively dialyzed. Deguanidination reaction of arginines was performed in aqueous solution by addition of 1 vol. of hydrazine to 0.1 mL of 10 mg/mL of H1 histone followed by heating at 75°C as described by Carr *et al.* (1981) but the reaction time was decreased to 15 s. All products were analyzed on 18% SDS/PAGE as reported by De Petrocellis *et al*. (1980) loading about 20 μg of protein in each slot. Unreacted protein aminogroups were titrated with 2,4,6-trinitrobenzenesulfonic acid (Wang, 1976).

Gel filtration chromatography. Native and modified H1 histones were gel filtered on 0.5 × 35 cm Sephadex G-100 columns equilibrated and eluted with 100 mM NaCl, 10 mM Tris/HCl, pH 7.2. Samples loaded in the gel filtration experiments contained 200 μg of histone in 0.02 mL of the same buffer. Fractions of 0.1 mL were collected at a flow rate of 0.5 mL/h. Elution profiles were determined with Coomassie blue staining according to Bradford (1976).

Velocity of sedimentation analyses. Ultracentrifugation studies were performed on 5 mL of 5–20% sucrose gradients in the presence of 0.1 M NaCl, 10 mM Tris/HCl, pH 7.2. Gradients were centrifuged at 39000 r.p.m. for 18 h at 18°C in SW 50.1 (Beckman). The distribution of protein in the gradients was determined by UV absorption at 230 nm.

CD spectroscopy. CD spectra, from 260 nm to 205 nm, were recorded on a Roussel Jouan CNRS III dichrograph (Jobin Yvon) and on a Jasco J500A spectropolarimeter equipped with a DP-500N data processor. Cells with 1.0 cm light path were used and the temperature was set at 22 ± 1°C. Values of data points at 222 nm, reported in the figures as indicative of overall structure, derive from duplicate spectra between 260 nm and 205 nm determined on a minimum of three independent H1 histone preparations. CD spectra were determined as a function of the concentration of sodium salts of chloride, phosphate and sulfate anions. The spectra of native and of differently modified H1 histones (0.1 mg/mL) were initially determined in distilled water. Then increasing amounts of each studied salt were added directly to the H1 solution in the cuvette to study the effect of the different anions on the protein structure.

RESULTS

The interaction of lysine aminogroups and arginine guanidino groups in the formation of H1 histone oligomeric states was studied by specific chemical modifications. Lysines were modified by carbamylation, acetylation or succinylation. Arginines were modified by deguanidination. As shown in Table 1, about 15% unreacted lysine aminogroups are present in modified H1 samples after acetylation or succinylation, 5% after carbamylation. About 20% of arginine residues remain unreacted after deguanidination with hydrazine. It is not possible to obtain better yields by hydrazinolysis because the increase of the reaction time or

Table 1. Content of modified residues in the derivatives of *C. variopedatus* sperm H1 histone utilized in the analyses.

Amino-acid modification	Modified residues (%)
Lysine acetylation	85
Lysine carbamylation	95
Lysine succinylation	85
Arginine deguanidination	80

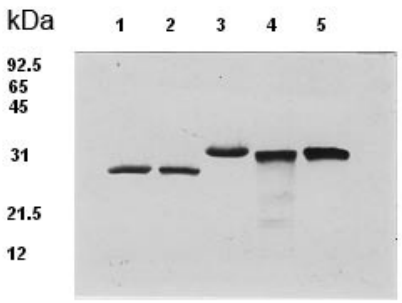

Figure 1. SDS/PAGE analysis of *C. variopedatus* sperm H1 histone derivatives.
Analysis on 18% SDS/PAGE of acetylated, carbamylated, succinylated, deguanidinated and native *C. variopedatus* sperm H1 histone (lanes 1–5), respectively.

reagent concentration increases rapidly fragmentation of the molecule, already apparent, although in a minor amount, in the optimized experimental conditions used here (Fig. 1, lane 4). The gel filtration profiles of native and modified H1 molecules in the presence of 100 mM NaCl are reported in Fig. 2. The native H1 histone shows an elution peak corresponding to about 65 kDa, a value clearly higher than that corresponding to its formula mass. Succinylated H1 is eluted in the column void volume indicating the formation of stable higher aggregated forms. Carbamylated H1 histone is eluted in a volume corresponding to the molecular mass of the monomer, about 22 kDa. It is evident that the gel filtration properties of the H1 histone molecule depend on the state of its lysine residues. When guanidino groups are removed from arginines by hydrazinolysis, ornithines are formed and the charge of the molecule remains the same because of the positively charged aminogroup of ornithine. The modification, however, causes a shift of the elution peak of H1 to values corresponding to a lower molecular mass but not as low as for the carbamylated molecule. It is possible that the non-complete deguanidination of arginines may add to the persistence of partial interactions producing a higher apparent molecular mass.

Electrophoretic analyses performed on native and modified H1 histone show the presence of only one component in all cases (Fig. 1). The observed minor differences in the mobility of the various modified forms with respect to the native molecule correlate well with the alterations in charge or molecular mass due to the chemical modifications. Deguanidination of arginines with formation of ornithines, although not altering the charge, does alter the ability of the molecule to form aggregates as shown by the gel filtration profile of the molecule (Fig. 2). This finding suggests that guanidino groups participate in the formation of oligomers of H1 and their substitution with aminogroups, as in the case of ornithines, modifies the properties of the molecule.

CD spectroscopy was used to study the influence of different ions on the structuring of native and of variously modified forms of *C. variopedatus* H1 histone. The ellipticity values at 222 nm (Fig. 3) were derived from spectra taken between 260 nm and 205 nm in the presence of increasing salt concentrations. It is evident that the modification either of the lysine amino groups or of the arginine guanidino groups of the H1 molecule result in a decreased ability of the histone to undergo structuring by salt addition. In particular, the succinylated H1 derivative shows CD spectra with [θ] values indicating partial structuring also in the absence of added salts and the value is not influenced by salt addition. This result is in line with the observed gel filtration properties that indicate the occurrence, in this modified H1 histone molecule, of stable high molecular mass aggregates (Fig. 2). The CD spectra indicate that the H1 histone, so modified, does not appear to require any intervening anions to stabilize intra/inter molecular interactions. The carbamylated H1 molecule is the derivative showing the minimum structuring differences with respect to the native form. It appears that the amido group formed

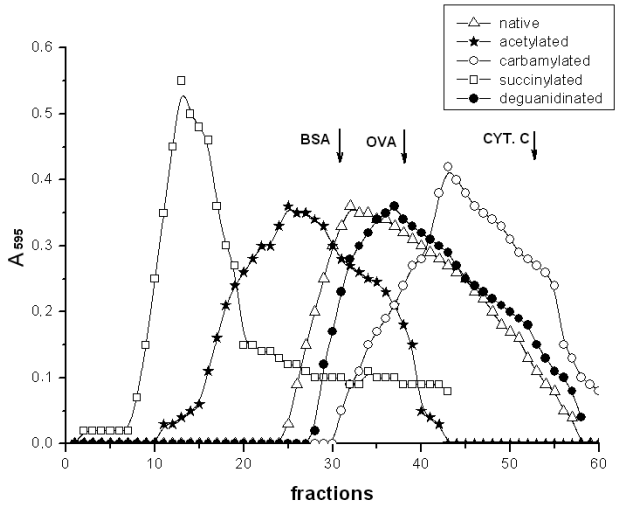

Figure 2. Gel filtration profiles of native and modified *C. variopedatus* sperm H1 histone.
Gel filtration analyses performed on Sephadex G-100 column, equilibrated with 100 mM NaCl, 10 mM Tris/HCl, pH 7.2. Arrows show the position of peak elution of the indicated marker proteins: BSA, bovine serum albumin (65 kDa), OVA, chicken ovalbumin (45 kDa), CYT. C, cytochrome *c* from horse heart (13 kDa).

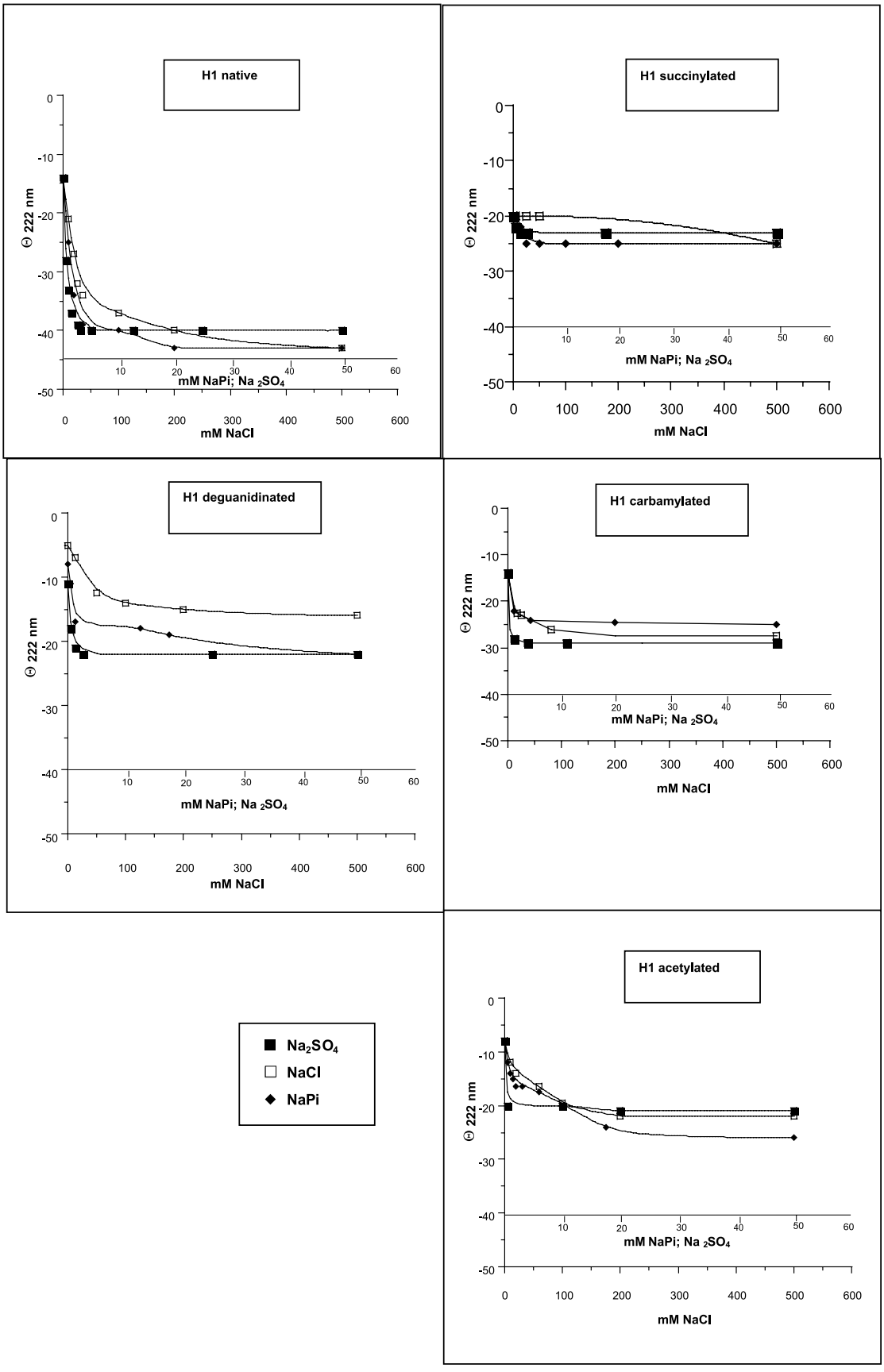

Figure 3. Circular dichroism spectra of native and modified *C. variopedatus* sperm H1 histone as a function of salt concentration.

CD spectra, from 260 nm to 205 nm, determined as a function of the concentration of sodium salts of chloride (NaCl), phosphate (NaP_i) and sulfate (Na_2SO_4) anions.

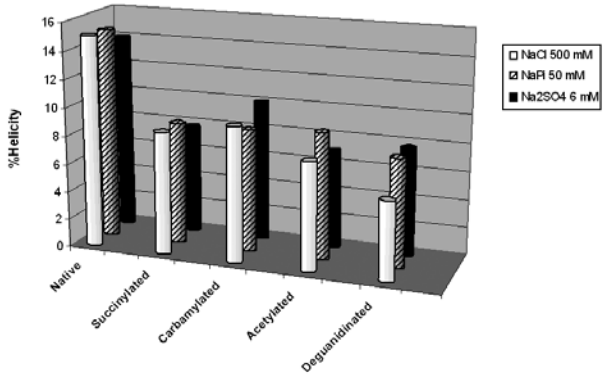

Figure 4. Helicity of native and modified *C. variopedatus* H1 histone as derived from CD spectra of the histones between 260 and 205 nm.

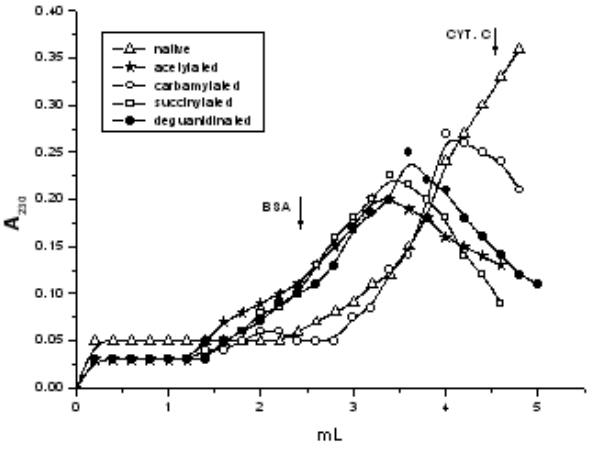

Figure 5. Sedimentation profiles on sucrose gradients of native and modified *C. variopedatus* sperm H1 histone. Analyses performed on 5–20% sucrose gradients in 100 mM NaCl, 10 mM Tris/HCl, pH 7.2. Arrows show the position of the indicated marker proteins: BSA, bovine serum albumin (65 kDa) and CYT. C, cytochrome *c* from horse heart (13 kDa).

by lysine reaction with potassium cyanate does not alter substantially the properties of the molecule. Since the [θ] value at 222 nm is the parameter usually taken as *indicative* of the overall structural organization of H1 histone molecules (Crane-Robinson *et al*., 1976; Giancotti *et al*., 1977; 1981; Barbero *et al*., 1980) and of histones H4 (Wickett *et al*., 1972) and H2B (D'Anna & Isenberg, 1972), we used a similar presentation. Assuming –1000° for random coil (Moss *et al*., 1976) and –30000° for α helix (Chen *et al*., 1974), the [θ] value at 222 nm of native *C. variopedatus* H1 histone molecule in distilled water corresponds to 15% helicity while the [θ] values at 222 nm of H1 histone derivatives correspond to values between 6 and 10% helicity. While the limit [θ] value, for native and modified H1 histone increases (except for the succinylated form) upon salt addition with a similar dependence for chloride ion and for ions with tetrahedral geometry, such as phosphate and sulfate, these latter ions induce the H1 helical structure at much lower concentrations than chloride (Fig. 4). The values of salt concentration that cause 50% maximum delta theta at 222 nm of native and modified H1 histone (C50%) taken as indicative of their structuring efficiency show, both for native and modified H1 histones, that sodium salts of chloride, phosphate and sulfate ions have increasing structuring effectiveness (Table 2). Velocity of sedimentation analyses on sucrose gradients (Fig. 5) demonstrate that native H1 has a sedimentation profile corresponding to a lower molecular mass value. The modified H1 forms all sediment faster than the native molecule and the carbamylated derivative is the only one showing a value corresponding to that of the formula mass value.

Table 2. Structuring efficiency of native and modified sperm *C. variopedatus* H1 histone by different anions.

H1 Derivatives	Cl^-	P_i	SO_4^{2-}
	C50% mM		
Native	18	1.19	0.49
Acetylated	26	3	0.2
Carbamylated	12.38	0.59	0.12
Succinylated	n.d.	1.06	0.30
Deguanidynated	31.72	0.74	0.35

DISCUSSION

Native H1 histone shows the highest level of protein structuring. Modification either of the lysine amino groups or of the arginine guanidino groups results in a decreased ability of the histone to undergo structuring by salt addition. When the interaction between arginine and lysine residues is perturbed, as occurs in the variously modified derivatives in which either lysines or arginines are modified, other less specific interactions become relevant. Gel filtration analyses on the different derivatives show molecular mass values higher than the formula mass and different from the corresponding values determined by velocity of sedimentation analysis. Particularly, native H1 histone shows an elution profile of gel filtration corresponding to a tetrameric molecule while the pattern of sedimentation velocity corresponds to a molecule smaller than the monomer. These results may be due to the peculiar structure of the native H1 histone in which the central region, the limit peptide, has a globular structure and the N- and C-terminal tails are not structured, causing a gel filtration pattern inconsistent with that of velocity of sedimentation.

The dramatic differences in the properties between the native and the succinylated H1 derivative clearly indicate the critical role of the positive charges of the lysines. In the modified histone a negative group substitutes the positive lysine amino group and the molecule organization becomes independ-

ent of added ions. The very small elution volume observed by gel filtration studies is also indicative of the presence of high molecular mass aggregates. The presence of a [θ] value at 222 nm in distilled water corresponding to about 50% of the maximum value of the native molecule and substantially invariant upon addition of ions also indicates that the modified histone has a stable intrinsic organization that does not correspond to and is not present in the native molecule. Also the velocity of sedimentation consistently indicates the occurrence of stable aggregated forms. It is noteworthy that phosphate ions are by about an order of magnitude more efficient than chloride ions in inducing H1 structuring. This, together with the reported presence on the H1 molecule of high affinity binding sites for phosphate ions (De Petrocellis *et al*., 1986), suggests that the interaction of the H1 histone with the phosphate groups of DNA in chromatin may be an important factor not only for the stabilization of aggregated forms of H1 but also for the stabilization of the higher order structure of chromatin through the specific contribution of phosphate linking ions. Such a possibility is in line with results showing that DNA induces structuring of the H1 molecule. Our results provide an additional detail in the interaction of the phosphate groups of DNA. They suggest that interaction of DNA phosphates occurs not with one but with two specific and critical positive groups of the H1 histone. In a recent study, Lu and Hansen (2004) showed that the ability of histone H1° to stabilize chromatin folding was not evenly distributed on the molecule; rather, it was confined to two specific subdomains in the CTD. Because the distribution of the positively charged lysine and arginine side chains is very similar throughout the about 100 amino acid-long C terminus domain, binding does not correlate in a simple manner with the abundance of positively charged amino acids within that region. Those authors proposed that H1 histone initially binds to DNA largely with low specificity, probably through the globular domain. In a second step, interaction would concern positive groups of the C terminus of the protein molecule that then would acquire, as a consequence, secondary structures (Lu & Hansen, 2004). The phosphate-mediated arginine–lysine side chain interactions might then contribute to this consequential structuring of H1 histone. In agreement with this possibility, it is particularly interesting that structuring of the acetylated H1 histone derivative, in the presence of phosphate, occurs at a C50% value higher than that of native and of other modified forms of the histone (Table 2). This result indicates that this modification, which mimics the *in vivo* acetylation, is the most efficient to decrease the capacity of phosphate to structure the H1 histone molecule. Consequently acetylation is the most efficient modification to destabilize the higher order chromatin folding and induce the transition from compact to open chromatin structure.

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