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Self-association of *Chaetopterus variopedatus* sperm histone H1-like. Relevance of arginine content and possible physiological role

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Self-association of histones H1 from calf thymus and from sperm of the marine worm *Chaetopterus variopedatus* was studied on native and glutaraldehyde cross-linked molecules by PAGE and by salt-induced turbidity measurements. Multiple polymers were generated by native sperm histone H1-like after glutaraldehyde cross-linking while the same treatment on its lysine- or arginine-modified derivatives and on somatic histone H1 failed to induce polymerization. This result suggests the relevance of arginine content in the formation of histone H1-like polymers particularly because *Chaetopterus variopedatus* and calf thymus histones H1 have similar content of lysine but different K/R ratio (2 and 15, respectively). Salt-induced turbidity experiments confirmed the high tendency of sperm histone H1-like to form oligomers, particularly in the presence of phosphate ions. Native PAGE analysis in the presence of phosphate supported this hypothesis. The reported results suggest that phosphate ions connecting lysine and arginine side chain groups contribute to the interaction of sperm histone H1-like with DNA in chromatin and play a key role in organization and stabilization of the chromatin higher order structures.

Keywords: *Chaetopterus variopedatus*, sperm histone H1-like, arginine

INTRODUCTION

Histones H1 are a family of very lysine-rich proteins that play a pivotal role in chromatin compaction. The basis for fiber compaction remains a matter of some debate, though protein–protein interactions may contribute significantly. Histone octamers (Dubochet & Noll, 1978) and nucleosomes have been reported to self-associate in solution (Ali & Singh, 1987; Segers *et al*., 1991). Particularly intriguing is the finding that self-association of nucleosomes or small oligonucleosomes is greatly enhanced by the addition of linker histones (Grau *et al*., 1982; Segers *et al*., 1991).

To date, attempts to determine whether linker histones specifically self-interact in the absence of DNA or chromatin have led to conflicting results from different research groups (Thomas *et al*., 1992; Draves *et al*., 1992; Maman *et al*., 1994). In order to clarify this point we have analyzed histone H1 self-association by biochemical and biophysical approaches. The studies were performed on histone H1-like from sperm of the marine annelid worm *Chaetopterus variopedatus (Ch.v.)* and on somatic calf thymus (C.T.) histone H1. Sperm histones H1-like have been identified in a wide range of organisms, including invertebrates (Ausiò, 1992), amphibians (Kasinsky *et al*., 1985; Itoh *et al*., 1997), and fish (Saperas *et al*., 1994; Watson & Davies, 1998).

Histones H1-like that mediate sperm-specific chromatin compaction contain higher amounts of arginine and similar lysine content in comparison to

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Abbreviations: APS, ammonium persulfate; *Ch.v., Chaetopterus variopedatus;* C.T., calf thymus; GTA, glutaraldehyde; BSA, bovine serum albumin; NaPi, sodium phosphate.

the somatic C.T. histone H1. In general, the K/R ratio of somatic histones H1 is higher than 15 while that of sperm histones H1-like is about 2 (De Petrocellis *et al*., 1983).

Although the C-terminal tails of somatic H1s are composed of 30–40% lysine and no arginine, the C-terminal tail of the sperm-specific histone H1-like of a sea urchin (Strickland *et al*., 1980) contains 44.3% lysine and 8.4% arginine (Lewis *et al*., 2004). Sperm *Ch.v.* histone H1 can be classified as histone H1-like on the base of its amino-acidic composition (De Petrocellis *et al*., 1983). We have already showed by gel filtration analysis that native *Ch.v*. histone H1-like shows tendency to form aggregates (Piscopo *et al*., 2006). Moreover, we have reported evidence that *Ch.v*. histone H1-like interaction with DNA occurs through phosphates connecting lysine and arginine side chain groups (Piscopo *et al*., 2006).

In this work we have investigated the role of these amino-acid residues and phosphate ions on the self-association of histones *Ch.v*. H1-like and C.T. H1. To this aim we performed specific chemical modifications of lysines or arginines of *Ch.v*. histone H1-like and analyzed their effects on salt-induced turbidity and gel electrophoretic mobility after treatment with the glutaraldehyde (GTA) cross-linking agent. The results provide new insight into these histone H1-like interactions.

MATERIALS AND METHODS

Materials. Chemical reagents and C.T. histone H1 were obtained from Sigma (USA).

Preparation of *Chaetopterus variopedatus* histone H1. *Ch.v.* sperm histone H1-like was purified as described by De Petrocellis *et al*. (1983). The purity of the protein was analysed by 18% SDS/PAGE and by acetic acid urea electrophoresis (AU/PAGE) (Panyim & Chalkley, 1969). The protein concentration was determined on the basis of the known tyrosine content: one tyrosine in C.T. H1 molecule (Johns, 1971) and two tyrosines in *Ch.v.* sperm H1 molecule (De Petrocellis *et al*., 1983) using $\varepsilon_{276} = 1340$ cm^{-1} M^{-1} (Giancotti *et al*., 1981).

SDS/PAGE analysis. Gels were run in vertical slab gel units (Mini Protean Biorad). Stacking gels consisted of 5.0% (w/v) acrylamide (acrylamide:bisacrylamide 30:0.15), 125 mM Tris, pH 6.8, 0.1% (w/v) SDS, 0.025% (w/v) ammonium persulfate (APS), and 0.025% (v/v) TEMED. Separating gels consisted of 18.0% (w/v) acrylamide (acrylamide: bisacrylamide 30:0.15), 750 mM Tris, pH 8.8, 0.1% (w/v) SDS, 0.025% APS, and 0.025% TEMED.

The electrode buffer consisted of 0.38 M glycine, 0.05 M Tris and 0.1% (w/v) SDS. Gels (83×58×0.75 mm) were run for 60 min at 100 V. Gels were fixed with 30% methanol, 10% acetic acid and stained using 0.2% Coomassie Brilliant Blue, 30% methanol, 10% acetic acid and destained with 30% methanol, 10% acetic acid.

Amino-acid side chain modifications. Acetylation and deguanidination reactions were performed according to Piscopo *et al.* (2006). Unreacted amino groups were titrated with 2,4,6 trinitro-benzene-sulfonic acid (Wang, 1976).

Histone H1 cross-linking by GTA. Native histones H1 and modified derivatives of *Ch.v.* histone H1-like were dissolved in 2 mM Tris/acetate buffer, 20 mM NaCl, pH 6.5, to obtain a final protein concentration of 0.25 mg/mL. The cross-linking reaction was performed at room temp. with 0.01% GTA, pH 8. Cross-linking was stopped after 30 min by adding 1 M urea; 7.5 μg of protein was analyzed on 18% SDS/PAGE.

Salt-induced turbidity analysis of H1 histones. All histones were assayed at 0.85 mg/mL, and BSA (Sigma) at 2 mg/mL. The optical density of the samples was measured at 420 nm (OD_{420}); we will refer to this optical density as turbidity. If $OD_{420} > 0.02$, the sample was centrifuged for 30 min at 13000 r.p.m. and 4°C in a microcentrifuge to remove aggregates. Samples were then brought to 1 mM sodium phosphate (NaPi), pH 7.2, by adding 100 mM NaPi, pH 7.2, dropwise, followed by vigorously pipeting the solution up and down. OD_{420} was then measured to detect aggregation. The aggregation effect of NaCl and NaPi on the above mentioned proteins was analyzed by increasing the salt concentration in 50 mM NaCl increments by adding 5 M NaCl dropwise to samples and in 5 mM NaPi increments by adding 400 mM NaPi, pH 7.2, dropwise to samples and then incubating the samples on ice for 15 min. As controls, BSA and saline solutions without histone proteins were used.

Native PAGE of H1 histones. Native H1 histones and modified derivatives of *Ch.v.* histone H1-like were analyzed by electrophoresis on 18% polyacrylamide slab gel in 50 mM Tris/phosphate buffer, pH 7.5, or on 12% polyacrylamide slab gel in 50 mM Tris/acetate buffer, pH 7.5. Native gel electrophoresis was performed using minigels (Bio-Rad). Each sample (10 μg) contained loading buffer (50 mM Tris/phosphate, pH 7.5, or 50 mM Tris/acetate buffer pH 7.5, 25% glycerol (v:v) and 0.001% pyronine (w:v)). Gels were run at 150 V for 3 h (18% acrylamide) or 90 min (12% acrylamide) at 4ºC. Proteins were observed after staining with Coomassie Brilliant Blue.

RESULTS

Figure 1 shows the electrophoretic pattern on AU/PAGE of *Ch.v.* and C.T. histones H1 used in the

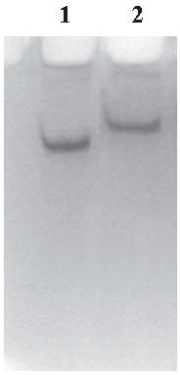

Figure 1. AU/PAGE analysis of native H1 histones.
Lane 1, *Ch.v.* histone H1-like; Lane 2, C.T. histone H1.

reported experiments (lanes 1 and 2, respectively). Both samples show single bands indicating their purity.

The electrophoretic pattern on 18% SDS/PAGE of native histones *Ch.v.* H1-like and C.T. H1 (Fig. 2, lanes 1 and 3) shows the different ability of these two types of histones to form oligomers. In fact, three bands corresponding to monomer, dimer and trimer are evident for *Ch.v.* histone H1-like (lane 1), while only monomer and dimer for C.T. histone H1 (lane 3). The higher molecular mass bands (about 50 kDa) revealed by SDS/PAGE in the native samples are oligomers likely to resist the denaturing conditions. In fact we have already reported anion-mediated lysine–arginine interactions (Piscopo *et al.*, 1993) and the sulfate anion of SDS may mediate this kind of interaction in SDS/PAGE.

In order to analyze the role of lysine and arginine side chain groups in the self-association of the sperm histone H1-like, we have modified both residues: lysines by acetylation and arginines by deguanidination. Almost 15% unreacted lysine amino groups are present in acetylated histone H1 while almost 20% arginine residues remain unreacted after deguanidination with hydrazine.

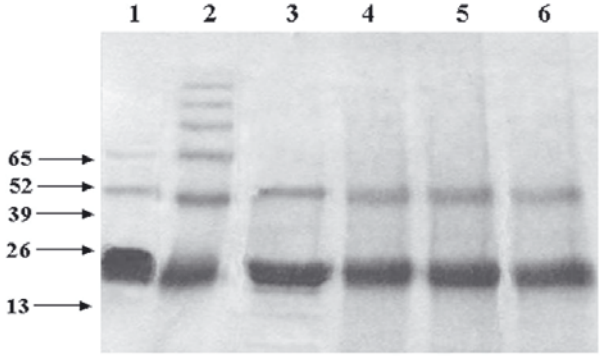

Figure 2. SDS/PAGE analysis of GTA-cross-linked native and acetylated H1 histones.
Lane 1, untreated native *Ch.v.* histone H1-like; Lane 2, GTA-cross-linked native *Ch.v.* histone H1-like; Lane 3, untreated native C.T. histone H1; Lane 4, GTA-cross-linked native C.T. histone H1; Lane 5, untreated acetylated *Ch.v.* histone H1-like; Lane 6, GTA-cross-linked acetylated *Ch.v.* histone H1-like.

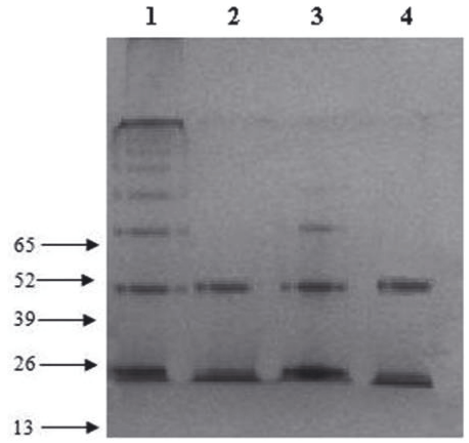

Figure 3. SDS/PAGE analysis of GTA-cross-linked native and deguanidinated H1 histones.
Lane 1, GTA-cross-linked native *Ch.v.* histone H1-like; Lane 2, GTA-cross-linked deguanidinated *Ch.v.* histone H1-like; Lane 3, untreated native *Ch.v.* histone H1-like; Lane 4, untreated deguanidinated *Ch.v.* histone H1-like.

Cross-linking reactions with GTA on native C.T. histone H1 and native and acetylated *Ch.v.* histones H1-like were performed and products analysed by PAGE (Fig. 2). It is evident that GTA-cross-linked native *Ch.v.* histone H1-like (lane 2) shows six bands ranging from monomer to hexamer while GTA-cross-linked C.T. H1 (lane 4) shows only two bands corresponding to monomer and dimer, as for the untreated molecule (lane 3). The same result as obtained with C.T. histone H1 is observed for acetylated *Ch.v.* histone H1-like derivative (Fig. 2, lanes 5 and 6) and for deguanidinated *Ch.v.* histone H1-like derivative (Fig. 3, lanes 2 and 4), suggesting that these modifications affect histone H1-like polymerization ability. For comparison GTA-cross-linked and untreated native *Ch.v.* histones H1-like are shown in lanes 1 and 3 of Fig. 3, respectively.

Results of salt-induced turbidity analyses on histones H1 in the presence of NaCl and NaPi are showed in Figs. 4 and 5, respectively. Turbidity analyses were also performed with saline solutions in the absence of proteins to verify that the increase of turbidity was indeed protein-related. The results indicate that up to 50 mM NaCl (Fig. 4) native and modified *Ch.v.* histones H1-like and native C.T. histones H1 show the same level of turbidity that is higher than that of BSA or acetylated C.T. histone H1. At higher salt concentrations the effect on turbidity is different for each molecule. In particular native sperm histone H1-like produces considerably more salt-induced turbidity than its derivatives or somatic histone H1. The acetylated C.T. histone H1 shows lower turbidity than that of the native molecule. The results of the experiments in the presence of NaPi (Fig. 5) show that this ion has an effect on

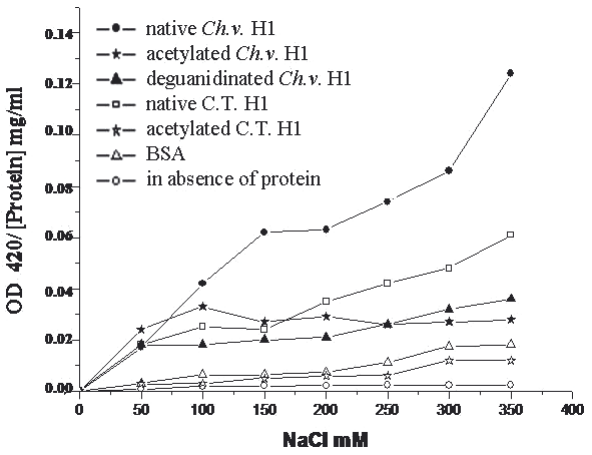

Figure 4. Salt-induced turbidity analysis of native and modified H1 histones in presence of NaCl.
Proteins were brought to 1 mM sodium phosphate (NaPi) pH 7.2, and titrated to increasing ionic strengths by adding NaCl in 50 mM increments. Turbidity was measured by optical density at 420 nm (OD_{420}) and all values are adjusted to reflect dilution by the addition of NaCl stock and normalized by protein concentration.

turbidity but at ten times lower concentrations than NaCl, indicating that phosphate is more efficient in inducing histone H1 aggregation. Also in this case C.T. histone H1 shows only a small increase in turbidity at increasing salt concentration. Acetylated and deguanidinated *Ch.v.* histones H1-like show a similar slight increase in turbidity until 5 mM NaPi that remains almost constant at higher salt concentrations. Thus both modifications reduce the formation of aggregates. The acetylated C.T. histone H1 shows lower turbidity than that of native molecule.

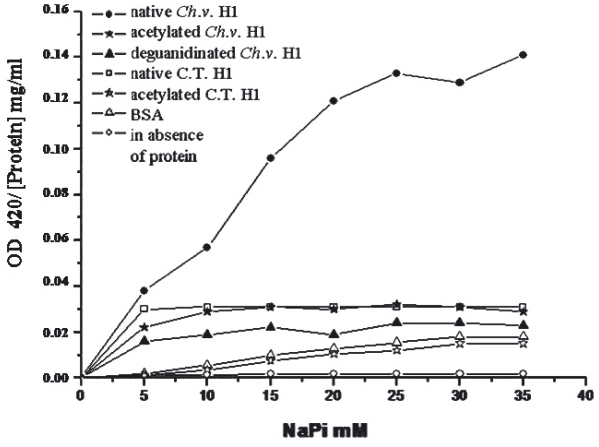

Figure 5. Salt-induced turbidity analysis of native and modified H1 histones in presence of NaPi.
Proteins were brought to 1 mM sodium phosphate (NaPi) pH 7.2, and titrated to increasing ionic strengths by adding NaPi in 5 mM increments. Turbidity was measured by optical density at 420 nm (OD_{420}) and all values are adjusted to reflect dilution by the addition of NaPi stock and normalized by protein concentration.

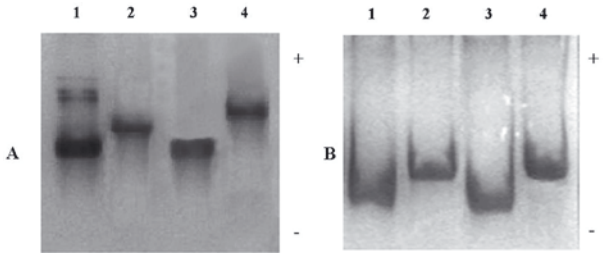

Figure 6. Native PAGE analysis in 50 mM Tris/phosphate, pH 7.5 (A) and in 50 mM Tris/acetate, pH 7.5 (B) of native and modified H1 histones.
Lane 1, native *Ch.v.* histone H1-like; Lane 2, native C.T. histone H1; Lane 3, deguanidinated *Ch.v.* histone H1-like; Lane 4, acetylated *Ch.v.* histone H1-like.

In order to confirm that phosphate groups, acting as bridge between lysine and/or arginine side chains, promote the formation of *Ch.v.* H1-like aggregates we performed native PAGE in 50 mM Tris/phosphate buffer, pH 7.5, and in 50 mM Tris/acetate, pH 7.5, of native and modified histones (Fig. 6). In the presence of phosphate (Fig. 6A), *Ch.v.* histone H1-like (lane 1) forms oligomers while C.T. histone H1 (lane 2) and *Ch.v.* histones H1 derivatives modified by deguanidination or acetylation are unable to form them (lanes 3 and 4). All the molecules failed to form aggregates in the presence of acetate (Fig. 6B). Proteins with modified arginines migrate with similar mobility as native *Ch.v.* H1-like does because deguanidination converts arginine in ornithine and the charge of the molecules remains the same.

DISCUSSION

Previous studies have indicated that linker 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). However, there has been controversy about whether self-association also occurs in solution and what is its molecular mechanism. Our results establish that sperm *Ch.v.* histone H1-like can undergo self-association in solution, as demonstrated by salt-induced turbidity and PAGE analyses. The somatic native and acetylated histone H1 and the acetylated and deguanidinated derivatives of sperm *Ch.v.* histone H1-like have a reduced ability to form aggregates.

In the salt-induced turbidity assay, NaPi is more efficient in inducing histone H1 aggregation than NaCl. These results confirm our previous studies in which we reported gel filtration and velocity of sedimentation analyses on native and on lysine- and arginine-modified derivatives of the sperm *Ch.v.* histone H1-like: anion-mediated lysine/arginine in-

teractions play a substantial role in the stabilization of the oligomeric states of the molecule (Piscopo *et al*., 2006). CD spectroscopy showed that phosphate anions are at least an order of magnitude more efficient than chloride as negatively charged groups connecting H1 lysines and arginines (Piscopo *et al*., 2006). On this basis we have proposed that DNA phosphates may be sandwiched between the lysine and arginine groups of histone H1 when this molecule binds to chromatin, constituting a relevant parameter for the reciprocal stabilization of the protein and chromatin higher order structures. These conclusions are also supported by native PAGE in the presence of phosphate (Fig. 6A). Since the molecule of *Ch.v.* sperm histone H1 is pure, the reduced mobility bands in the native PAGE (Fig. 6A) probably correspond to oligomers formed by interaction between lysine and arginine chain groups mediated by phosphate anion, as suggested by Piscopo *et al*. (2006). Then native *Ch.v*. H1-like aggregates while native C.T. H1 does not because its K/R ratio is 15; moreover, both derivatives of *Ch.v*. histones H1-like fail to aggregate because they lack almost completely arginine or lysine.

Experiments of GTA cross-linking of *Ch.v.* sperm histone H1-like showed the formation of a broad spectrum of oligomers and a lower capacity for aggregation of C.T. histone H1. These results are in agreement with those obtained by Russo *et al*. (1983) with another cross-linking agent and with those reported by McIntosh (1992), suggesting an involvement of monomeric di-aldehyde and a lysyl and an arginyl residue in the stable cross-link. Since histones C.T. and sperm *Ch.v.* H1-like differ substantially only in their arginine content (1.8 mol and 12.6 mol percentage, respectively), probably the ability to generate oligomers larger than dimer could be due to the interaction of the guanidinum group of arginines with the amino group of lysines in *Ch.v.* histone H1-like. In fact, the acetylated derivative that has few free amino groups of lysine, forms no more than a dimer probably because of the reduced number of interactions with arginine. The deguanidinated derivative shows the same behaviour as the acetylated one. Moreover, it behaves similarly to the somatic histone H1 because the modification of 80% arginines, with the formation of ornithine, produces molecules that have a K/R ratio similar to that of C.T. histone H1.

In conclusion, these three experimental approches indicate a higher ability of *Ch.v.* sperm histone H1-like to form aggregates in comparison with that observed for the somatic histone H1. Our results are in accordance with those reported by Carter and van Holde (1998), who established by turbidity and cross-linking studies that avian-specific linker histone H5 can undergo self-association in solution. Interestingly, the K/R ratio of this histone is similar to that of sperm *Ch.v.* histone H1-like.

Sperm DNA is more highly condensed that the DNA in mitotic chromosomes. To achieve this high degree of packaging, sperm DNA interacts with protamines to form linear, side by side arrays of chromatin or with protamine-like type proteins and histones (Lewis & Ausiò, 2002). This differs markedly from the looser DNA packaging of somatic cell nuclei and mitotic chromosomes, in which the DNA is coiled around histone octamers to form nucleosomes.

In summary, this enhanced ability of sperm *Ch.v*. histone H1-like to form oligomeric structures suggests its central role in the high condensation of sperm chromatin.

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