Does High-Mobility-Group Non-Histone Protein HMG 1 Interact Specifically with Histone H1 Subfractions?

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The interaction of the non-histone chromosomal protein HMG (high-mobility group) 1 with histone H1 subfractions was investigated by equilibrium sedimentation and n.m.r. spectroscopy. In contrast with a previous report [Smerdon & Isenberg (1976) *Biochemistry* 15, 4242-4247], it was found, by using equilibrium-sedimentation analysis, that protein HMG 1 binds to all three histone H1 subfractions CTL1, CTL2, and CTL3, arguing against there being a specific interaction between protein HMG 1 and only two of the subfractions, CTL1 and CTL2. Raising the ionic strength of the solutions prevents binding of protein HMG 1 to total histone H1 and the three subfractions, suggesting that the binding *in vitro* is simply a non-specific ionic interaction between acidic regions of the non-histone protein and the basic regions of the histone. Protein HMG 1 binds to histone H5 also, supporting this view. The above conclusions are supported by n.m.r. studies of protein HMG 1/histone H1 subfraction mixtures. When the two proteins were mixed, there was little perturbation of the n.m.r. spectra and there was no evidence for specific interaction of protein HMG 1 with any of the subfractions. It therefore remains an open question as to whether protein HMG 1 and histone H1 are complexed together in chromatin.

Although considerable advances have been made recently in our understanding of histone interactions in chromatin, little is known at present about the interaction of the many non-histone chromosomal proteins. This is due partly to the fact that few pure non-histone proteins have been isolated in sufficient quantity to allow detailed analysis *in vitro*. One group of non-histone proteins that has been reasonably well characterized over the last few years is the HMG proteins (Johns *et al.*, 1975). These have been fractionated and the four main components, proteins HMG 1, 2, 14 and 17, have been characterized (Goodwin *et al.*, 1978a). The interactions of these proteins with DNA and histones have been studied in order to gain some insight into their chromosomal function(s) (Shooter *et al.*, 1974; Goodwin *et al.*, 1975a; Cary *et al.*, 1976; Yu *et al.*, 1977; Javaherian & Wang, 1978; Abercrombie *et al.*, 1978; Javaherian & Amini, 1977; Smerdon & Isenberg, 1976; Yu & Spring, 1977).

Protein HMG 1 was found to bind to both DNA, (Shooter *et al.*, 1974; Cary *et al.*, 1976; Yu *et al.*, 1977; Javaherian & Wang, 1978) and to histone H1

(Shooter *et al.*, 1974). This interaction with histone H1 has been examined in more detail by Smerdon & Isenberg (1976), and they have reported a specificity in this interaction in that calf thymus protein HMG 1 appeared to interact specifically with only two of the histone H1 subfractions, subfractions CTL1(b) and CTL2, forming heterodimers with each. Similarly, Yu & Spring (1977) have found that HMG 1 binds to the different histone H1 subfractions in the order CTL2 > CTL1 > CTL3.

In view of the importance of such a putative specific interaction in the study of chromatin structure, we have also been studying the protein HMG 1/histone H1 interaction by using ultracentrifugation and n.m.r. techniques. The present paper reports the results of this investigation.

Experimental

Preparation of histones and HMG proteins

Calf thymus histone H1 was extracted by 5% HClO₄ (Johns, 1964). The subfractions were separated by the method of Kinkade & Cole (1966). The experimental details were given previously (Bradbury *et al.*, 1975). No attempt was made to separate subfraction CTL3 into further components.

Abbreviations used: HMG protein, high-mobility-group protein; subfractions CTL1, CTL2 and CTL3, subfractions of the lysine-rich histone H1.

Chicken erythrocyte histone H5 was prepared by the method of Johns & Diggle (1969). Proteins HMG 1 and HMG 2 were prepared from calf thymus as described previously (Goodwin *et al.*, 1975b).

Ultracentrifugation studies

All proteins were weighed out and initially dissolved in 10mM-HCl. After 2–4h at room temperature the solutions were separately dialysed overnight at 4°C against the appropriate buffer. Concentrations of these solutions were estimated from the number of fringes observed interferometrically in a synthetic boundary cell in the analytical ultracentrifuge. Appropriate volumes of the solutions were mixed and, without further dialysis, the molecular weights measured by the low-speed equilibrium method as described previously (Shooter *et al.*, 1974). Centrifugation was carried out at 8000–13 500 rev./min for 20–24h. The concentration of protein in these solutions was always close to 4mg/ml. Weight-average molecular weights ($\bar{M}_w$) were calculated from the observed concentration difference from the meniscus to the bottom of the centrifuge cell.

N.m.r. spectroscopic studies

Calf thymus histone H1 subfractions and protein HMG 1 were dissolved separately in $^2\text{H}_2\text{O}$ at pH 3. The appropriate protein solutions were then mixed and dialysed against $^2\text{H}_2\text{O}$ /phosphate buffers with NaCl added where indicated. For the DNA-recombination experiments, the histone H1/protein HMG 1 mixtures were dialysed against $^2\text{H}_2\text{O}$ /1M-NaCl/5mM-phosphate, pH 7.0. Calf thymus DNA (Sigma grade 1) was dissolved in the same medium. The DNA and protein solutions were mixed and dialysed in stages to the appropriate salt concentrations.

The ^1H n.m.r. spectra of all samples were recorded on a Bruker WH 270 spectrometer equipped with an Oxford Instruments superconducting magnet. Free induction decays were accumulated in double-precision arithmetic (G. E. Chapman, unpublished work), and between 1000 and 20 000 decays were accumulated for each sample. The residual water signal was eliminated by irradiation in the waiting period between free induction decays. The repetition period was 0.9s.

Results

Equilibrium-sedimentation studies

In the course of the present study the molecular weights of the individual components, HMG 1 protein and H1 histones, were measured by equilib-

rium sedimentation in the 10mM-phosphate, pH 7, buffer at different ionic strengths (0.1–1.0M-NaCl). Total histone H1 and subfraction molecular weights varied between 19 600 and 20 800. HMG 1 protein occasionally showed some aggregation after dialysis, and the molecular weight varied between 25 000 and 33 000. These values for histone H1 and protein HMG 1 molecular weights are reasonably close to those obtained from the sequence of histone H1 (21 600) (Cole, 1977) and from previous studies on the molecular weight of protein HMG 1, which gave a value of 26 500 (Shooter *et al.*, 1974). In the analysis of the results in the present paper we have used these two values, 21 600 and 26 500, for the molecular weights of histone H1 and protein HMG 1 respectively, so that a 1:1 dimer of the two proteins would have a mol.wt. of 48 100. On this basis a mixture of 1:1 (w/w) contains 1 mol of protein HMG 1:1.2 mol of histone H1.

The results of measurements of 1:1.2 (mol/mol) mixtures of protein HMG 1 and whole histone H1 are given in Table 1. In the absence of interaction, the expected $\bar{M}_w$ would be 24 000. If the interaction involved the formation of a 1:1 dimer and free excess histone, the molecular weight expected would be 46 000. The data of Table 1 show that in 0.1M- and 0.2M-NaCl the two proteins form complexes with molecular weights consistent with a histone H1–protein HMG 1 dimer, but that as the salt concentration is further increased, the extent of interaction decreases, and by 0.5M-NaCl the molecular weight corresponds to that expected for a mixture of monomer histone H1 and protein HMG 1 molecules. The data strongly suggest the formation of a dimer, histone H1–protein HMG 1, at low salt molarity, and this dimer dissociates into histone H1 and protein HMG 1 monomers as the ionic strength is increased to 0.5 mol/litre.

Table 1. *Interaction of protein HMG 1 and whole histone H1*
Weight-average molecular weights ($\bar{M}_w$) of 1:1.2 (mol/mol) protein HMG 1–histone H1 mixtures were measured at various salt concentrations, all buffered at pH 7.0 with 10mM-phosphate. The values given in parentheses give the molecular-weight ranges from the top to the bottom of the centrifuge cell. The total protein concentration was approx. 200 μM .

[NaCl] (M)	$\bar{M}_w$ of protein HMG 1–histone H1 complex
0.1	47 600 (35 000–60 000)
0.1	44 000 (30 000–53 000)
0.1	47 700 (35 000–62 000)
0.2	47 300 (39 000–58 000)
0.2	52 400 (46 000–62 000)
0.3	31 500 (27 000–36 000)
0.4	27 000 (25 000–32 000)
0.5	26 800 (25 000–30 000)

Table 2. Interaction of protein HMG 1 and subfractions of histone H1 in 0.1 M-NaCl/10 mM-phosphate buffer, pH 7.0

Histone subfraction	Molar ratio (protein HMG 1/histone H1 subfraction)	$\bar{M}_w$	
		Expected*	Observed†
CTL1	1:1.6	42 500	41 800 (41 800–41 800)
CTL1	1:4	32 900	30 500 (28 000–32 000)
CTL1	2.2:1	—	71 200 (64 000–84 000)
CTL2	1:1.2	46 000	51 600 (48 000–54 000)
CTL3	1:1.2	46 000	50 800 (48 000–53 000)
CTL3	3.6:1	—	75 400 (59 000–85 000)

* Estimates of the weight-average molecular weights ($\bar{M}_w$) were made by assuming the presence of a 1:1 dimer and excess free histone H1 subfraction.

† The values given in parentheses are the molecular-weight ranges from the top to the bottom of the centrifuge cell. The total protein concentration was approx. 200 μ M.

A trial experiment with a 1:1.2 (mol/mol) mixture of protein HMG 2 and histone H1 in 0.2 M-NaCl/10 mM-phosphate buffer, pH 7.0, gave a mol.wt. of 29 300, suggesting little interaction between these two proteins. This confirms the results of Smerdon & Isenberg (1976) and Yu & Spring (1977); both groups of workers found no interaction between protein HMG 2 and histone H1.

The interaction of protein HMG 1 with the subfractions of histone H1 has also been studied (Table 2). In the low-ionic-strength buffer used (0.1 M-NaCl/10 mM-phosphate) it was found that protein HMG 1 binds to all three subfractions, in contrast with the findings of Smerdon & Isenberg (1976), who reported that protein HMG 1 binds only to subfractions CTL1(b) and CTL2. The results of Table 2 also show that the addition of excess protein HMG 1 leads to the formation of aggregates of molecular weights higher than dimers, i.e. the protein HMG 1–histone H1 dimer can bind further protein HMG 1 molecules. In contrast, when histone H1 is in excess, as exemplified with subfraction CTL1, aggregation does not appear to go beyond the formation of a dimer.

The expected decrease in the extent of interaction with the histone subfraction with increasing ionic strength of the buffer was again confirmed, e.g., in 0.1 M-phosphate buffer 1:1.2 (mol/mol) mixtures of protein HMG 1 and subfractions CTL1 and CTL3 gave $\bar{M}_w$ values of 34 500 and 33 900 respectively, compared with the expected $\bar{M}_w$ of 46 000 for dimer plus free histone. In 1.0 M-NaCl/10 mM-phosphate buffer, pH 7.0, a 1:1.2 (mol/mol) mixture of protein HMG 1 and subfraction CTL2 was found to have a mol.wt. of 24 900, compared with the value of 24 000 expected in the absence of interaction.

The experiments described here thus suggest that protein HMG 1 does not bind in a highly specific manner with histone H1 subfractions. The fact that the interaction is readily destroyed on raising the ionic strength indicates that the interaction is

primarily ionic. One might then expect the protein HMG 1 molecule to bind to other basic proteins, and indeed we have found that it binds to histone H5; a 1:1.4 (mol/mol) mixture of protein HMG 1 and histone H5 in 0.1 M-NaCl/10 mM-phosphate, pH 7, had an $\bar{M}_w$ of 50 400.

N.m.r. spectroscopy studies

The n.m.r. spectra of subfractions of histone H1, HMG 1 and an equimolar mixture of the two were run under the following conditions: (a) 4×10^{-6} M and 4×10^{-5} M in 20 mM-phosphate, pH 7.6, as described by Smerdon & Isenberg (1976), and (b) 4×10^{-4} M in 0.1 M-NaCl/10 mM-phosphate, pH 7.0, under the conditions used in the present paper for the centrifugation studies. In the latter experiment it was found that protein HMG 1 at 4×10^{-4} M was in part aggregated, leading to line broadening in the protein HMG 1 spectrum. However, in the mixtures of histone H1 and HMG protein there is little aggregation. The histone H1 molecules appear therefore to interact with the HMG protein, preventing them from aggregating. Reproducible n.m.r. results were obtained for all complexes of the HMG proteins with histone H1 subfractions.

Fig. 1 gives a representative set of spectra; the only feature indicating complex-formation between histone H1 subfractions and protein HMG 1 appears to be the slight broadening or loss of peak height of the perturbed resonances both in the aromatic (6–8 p.p.m.) and apolar upfield (1 to –0.5 p.p.m.) regions. Although peak broadening is expected on dimer formation because of the increase in tumbling time of the complex compared with the monomers, the effect observed in Fig. 1 is smaller than would be expected for a globular complex of mol.wt. 50 000. Structured regions in histone H1 and protein HMG 1 are clearly indicated by the complexity of the aromatic spectrum and the presence of perturbed upfield-shifted resonances, the ring-current-shifted

Fig. 1. 270 MHz Fourier spectra of equimolar mixtures (4×10^{-5} M) of protein HMG 1 and histone H1 and histone H1 subfractions (b) compared with spectra obtained by summing the spectra of the separate components (a)

(i) Protein HMG 1+total histone H1; (ii) protein HMG 1+subfraction CTL1; (iii) protein HMG 1+subfraction CTL2; (iv) protein HMG 1+subfraction CTL3. Eightfold expansions of the aromatic (6–8 p.p.m.) and upfield (1 to –1 p.p.m.) regions are also shown. (In some spectra, trace contamination by acetone causes a sharp resonance at 2.3 p.p.m.). Spectra were obtained in 20 mM-phosphate buffer, pH 7.6. The n.m.r. conditions were the same as those used by Smerdon & Isenberg (1976).

resonances from apolar residues constrained to lie close to aromatic rings. In addition, the sharpness of many resonance peaks strongly suggests flexible mobile regions of histone H1 and protein HMG 1. This is consistent with the conclusions that histone H1 in its structured form contains a globular central region from residues 41 to 116 and non-structured mobile *N*- and *C*-terminal regions. The spectra of the histone H1–protein HMG 1 complexes shown in Fig. 1(b) do not contain any evidence for a strong complex that modifies the characteristics of the structured regions of the individual proteins. The chemical-shift values for the perturbed apolar resonances remain constant when the complexing occurs (this is true for all histone H1 subfraction/protein HMG 1 mixtures). Hence we can say that the interior of the globular structure of both proteins is unaffected by the formation of dimers. It is probable that the interaction between the two molecules does not involve apolar interaction, but is primarily ionic between charged lysine residues, and glutamic acid and aspartic acid residues in flexible regions of histone H1 and HMG 1 protein. This is consistent with the n.m.r. results in high salt (greater than 0.5 M; results not shown) when the perturbed broadened resonances return to their former line-widths and the spectrum of the complex is exactly the sum of the spectra of the individual components. We cannot at present determine by n.m.r. experiments whether the complex-formation is a specific electrostatic interaction; no differences in the chemical shift of the protons from the $-\text{CH}_2$ of

lysine (3.02 p.p.m.) or the $-\text{CH}_2$ of glutamic acid (2.3 p.p.m.) are observed. It is noteworthy in Fig. 1 that the spectrum of the subfraction CTL3 protein/HMG 1 mixture shows a sharper perturbed resonance at -0.26 p.p.m. compared with the spectra of subfractions CTL1 and CTL2 complexed with protein HMG 1. This suggests that the interaction is weaker between protein HMG 1 and histone H1 subfraction CTL3 under these conditions.

The interaction of the histone H1–protein HMG 1 complex with DNA was studied in order to look for cross-interactions of the proteins when bound to DNA. The n.m.r. spectra of histone H1–DNA complexes show considerable signal from histone H1 (Bradbury *et al.*, 1975), indicating that substantial regions of the molecule are not rigidly bound to the DNA. Similar results have been obtained for protein HMG 1–DNA complexes (Cary *et al.*, 1976). The n.m.r. spectra of histone H1–DNA, protein HMG 1–DNA and histone H1–protein HMG 1–DNA complexes at low salt concentrations at a protein/DNA weight ratio of 1:5 were studied by n.m.r. with subfractions of histone H1. The sum of the spectra of histone H1–DNA and protein HMG 1–DNA complexes (results not shown) appear identical with the spectrum of the triple complex for each subfraction. There also appears to be no evidence of protein HMG 1 affecting the histone H1–complexing behaviour with DNA or vice versa, hence we can say there is no evidence of a strong cross-interaction of histone H1 with protein HMG 1 when bound to DNA.

Discussion

The present results demonstrate that, when protein HMG 1 and histone H1 are mixed in a low-ionic-strength buffer, a complex is formed, the composition of which appears to be determined by the input ratios of the two proteins. This interaction is observed with the three histone H1 subfractions as well as with total histone H1. On raising the ionic strength of the solution above 0.3 mol/litre the histone H1-protein HMG 1 complex dissociates, which suggests that the two proteins form a complex primarily through ionic interactions.

The n.m.r. data also show indications of complex-formation in that, at low-ionic-strengths (0.05–0.3 M-NaCl), there is some peak broadening of resonances, but in high salt these resonances return to the line-widths of the individual proteins also in high salt (0.3–1.0 M-NaCl), i.e. the spectrum of the histone H1/protein HMG 1 mixture at high salt, is equal to the sum of the individual spectra of histone H1 and protein HMG 1 under the same conditions. The n.m.r. data further suggest that the histone H1-protein HMG 1 dimer in low salt does not involve distortions of the interior of the globular regions of histone H1 and protein HMG 1 as there are no changes in the chemical shifts of the perturbed apolar or aromatic resonances. These perturbations result from the precise arrangement of aromatic and apolar residues within the globular regions, and any change in the tertiary structure of the globular units will result in a detectable frequency shift in these perturbed resonances, which is not observed. The suggestion is therefore that the dimers of histone H1 and protein HMG 1 retain the integrity of the structured globular regions of both histone H1 and protein HMG 1. It is also probable that the protein HMG 1-histone H1 complex does not form a compact globular dimer, because the n.m.r. spectra of the complex are not typical of a fully globular protein of a mol.wt. of 50000. This statement is supported by the finding that the c.d. data show no change of the α -helical and random-coil components on complex-formation (Smerdon & Isenberg, 1976). Further, the observation that the complex dissociates at low concentrations of the subfractions [this is particularly true for subfraction CTL3+protein HMG 1 as observed by n.m.r. and fluorescence measurements (Smerdon & Isenberg, 1976)] suggests a weak association of histone H1 and protein HMG 1.

Studies on the primary structures of protein HMG 1 have revealed a remarkable feature. The C-terminal region of the protein has an unbroken tract of about 40 residues that is composed almost entirely of aspartic and glutamic acid residues (Walker *et al.*, 1978). The protein HMG 1 molecule thus has a fairly basic N-terminal two-thirds and a highly acidic C-terminus. This acidic C-terminus of protein HMG 1 most probably binds to the basic

C-terminus of histone H1 when the two are mixed *in vitro*, forming a dimer with a net positive charge. This dimer could then bind further protein HMG 1 molecules if the latter is in excess. This explains the centrifugation data obtained with histone H1 subfraction CTL1 and protein HMG 1; when protein HMG 1 is in excess, high-molecular-weight aggregates are formed, but when histone H1 is in excess the mixture behaves as if composed of a histone H1-protein HMG 1 dimer plus free histone H1. Protein HMG 2, on the other hand, although having a primary structure similar to that of protein HMG 1 (Walker *et al.*, 1976), does not bind to histone H1. This is probably because, firstly, it is more basic than protein HMG 1 (for example, protein HMG 1 does not bind to CM-cellulose at pH9, whereas protein HMG 2 does) and, secondly, the C-terminal acidic region of protein HMG 2 is relatively inaccessible, since proteolytic cleavage of the C-terminus of protein HMG 2 occurs much less readily than does that of protein HMG 1 (Goodwin *et al.*, 1978b). Is the dimer interaction ionic only, involving the electrostatic interaction of the acidic and basic residues of protein HMG 1 and histone H1? This is a difficult question to answer, but we can say that the basic random-coil or extended-polypeptide N- and C-terminal regions of histone H1 have very few apolar residues and are rich in helix-destabilizing residues such as proline, glycine and serine. Hence it is highly unlikely that interactions between the basic and acidic regions of the two proteins will involve hydrophobic interactions of any significance. This leaves the possibility of hydrophobic interaction between the globular regions of histone H1 and protein HMG 1. There is, however, no change in the perturbed apolar and aromatic resonances in the n.m.r. spectrum of the complex of histone H1 and HMG 1 protein. If hydrophobic interactions were strong, they should be present in 0.3 M-NaCl, because the globular regions for both proteins are stable from 0.05 to 2.0 M-NaCl at pH7.0. Hence it is probable that the interactions between protein HMG 1 and histone H1 subfractions are primarily ionic.

Our finding that protein HMG 1 binds to all three subfractions of histone H1 and also to another histone, H5, would appear to argue against any specificity in the protein HMG 1-histone H1 subfraction interaction. Even in the presence of DNA, no evidence was obtained to suggest that protein HMG 1 binds specifically to any of the histone H1 subfractions. Also, by using n.m.r., we have not found any evidence for an interaction between trout testis histone H1 (which has no subfractions) and protein HMG-T, the trout testis protein equivalent to calf proteins HMG 1 and HMG 2 (Watson *et al.*, 1977).

Our conclusions, then, are that although thymus protein HMG 1 does bind to histone H1 *in vitro* (as

has been found previously), we have not found any evidence for a specificity in this interaction or any evidence to suggest that it occurs *in vivo*. Our results do not, of course, rule out the possibility that these two proteins are combined together in chromatin, and other approaches such as chemical cross-linking experiments on the native chromatin will be required to resolve this question.

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