COMPARISON OF THE SALT DISSOCIATIONS OF HIGH MOLECULAR WEIGHT HMG NON-HISTONE CHROMATIN PROTEINS FROM DOUBLE-STRANDED DNA AND FROM CHROMATIN

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1. Introduction

The high mobility group (HMG) proteins are well-characterized non-histone chromatin proteins first identified [1] in calf thymus. It has proved possible to identify proteins from phylogenetically diverse organisms that are clearly sequence homologs of the calf thymus HMG proteins and that, therefore, can reasonably be included in the HMG class. The HMG proteins fall into two distinct subsets. The first contains proteins with $M_r \sim 26\,000$: the calf thymus prototypes, HMG-1 and HMG-2 [1]; their direct analogs from other higher vertebrates [2,3]; HMG-E (or HMG-2a [4]), a prominent non-histone protein in chicken erythrocyte chromatin [2]; and HMG-T from trout testis [5]. We will refer to that group of proteins as the high molecular weight HMG proteins, since the second HMG subset contains proteins of substantially lower molecular weights: the sequence homologous calf thymus prototypes, HMG-14, M_r 11 000 [6], and HMG-17, of M_r 10 000 [7]; their direct analogs in other higher vertebrates [8,9]; and H-6 from trout testis [10].

The high molecular weight HMG protein from chicken erythrocytes, calf thymus and cultured rat hepatoma cells possesses preferential affinity for single-stranded DNA [11,12]. That property is potentially important in understanding the biological functions of the proteins, particularly in light of the fact that at 0.2 M NaCl (which presumably provides a roughly physiological ionic strength), the proteins appear to bind exclusively to single-stranded DNA [12].

Here we report a more detailed study of the NaCl dissociation of HMG-1, HMG-2 and HMG-E from double-stranded DNA, and compare those results with the NaCl-promoted release of the same proteins from chromatin. Our results indicate that binding of the high molecular weight HMG proteins in chromatin is rather different from the binding of the proteins to purified double-stranded DNA, and suggest that a major portion of the proteins might be bound to single-stranded DNA regions in chromatin.

2. Materials and methods

2.1. *Nuclei isolation and chromatin extractions*

Nuclei were isolated as in [13] from chicken erythrocytes (Pel-Freez) by repeated homogenization in 1% Triton X-100/0.25 M sucrose/50 mM Tris–HCl (pH 7.5)/3 mM $CaCl_2$/0.1 mM phenylmethane sulfonylfluoride. The nuclei were swollen to yield chromatin by homogenization in 1 mM Tris–HCl (pH 7.5). For DNA-cellulose chromatography, unsheared chromatin (A_{260} 20) was brought to 0.35 M NaCl and centrifuged at 25 000 × g for 30 min. The supernatant, which contained extracted proteins, was used to isolate HMG-1, HMG-2 and HMG-E.

For analyzing proteins that were released from chromatin at several ionic strengths, 4 ml aliquots of unsheared chromatin (A_{260} 16) in 10% sucrose/1 ml Tris–HCl (pH 7.5) were adjusted in centrifuge tubes to 0.08, 0.13, 0.20 or 0.35 M NaCl by adding solid NaCl. Underneath those samples were layered 8 ml portions of 20% sucrose/1 mM Tris–HCl (pH 7.5) containing the appropriate, corresponding NaCl levels. The samples were then centrifuged at 40 000 rev./min

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for 18 h with a Beckman SW41 rotor at 4°C. Compact pellets were formed in all tubes during centrifugation. For electrophoretic analysis of dissociated proteins, non-pelleted material was collected in fractions by pumping 60% sucrose into the bottom of each tube. For analysis of undissociated proteins, pellets were recovered from separate tubes by pouring off the sucrose solutions.

2.2. *DNA-cellulose chromatography*

Double-stranded DNA-cellulose was prepared from Avicell microcrystalline cellulose and salmon sperm DNA (Sigma type III) as in [14]. Single-stranded DNA regions were removed by treating the double-stranded DNA column with S_1 nuclease (Calbiochem) as in [11,12]. DNA column chromatography was done at 4°C.

A sample containing chicken erythrocyte HMG-1, HMG-2 and HMG-E was obtained by sequential chromatography done as in [12] on columns containing immobilized double-stranded and single-stranded DNA preparations. The 3 proteins were eluted together from a single-stranded DNA-cellulose column by applying 0.5 M NaCl/1 mM Tris–HCl (pH 7.5). That eluate contained essentially only HMG-1, HMG-2 and HMG-E, as expected (tracks 4, 5 in fig.3 of [12]).

2.3. *Gel electrophoresis*

Protein samples were analyzed by electrophoresis in the presence of SDS in gels polymerized from 15% polyacrylamide/0.2% bisacrylamide as in [15]. Protein samples from double-stranded DNA chromatography were concentrated 10-fold by ultrafiltration and prepared for electrophoresis by precipitation at 25% trichloroacetic acid and acetone washing. Gels of those samples were stained with Coomassie brilliant blue R as in [16].

Fractions containing dissociated proteins from sucrose centrifugations were combined as indicated by bars in fig.3. The proteins dissociated with 0.08 M and 0.13 M NaCl were concentrated 10-fold by coating dialysis bags containing the samples with Sephadex G-200. The proteins dissociated by 0.2 M and 0.35 M NaCl were not concentrated. All 4 samples were dialyzed against SDS gel electrophoresis sample buffer. Pellets obtained by centrifugation in 0.20 M and 0.35 M NaCl were suspended in 5 ml 5% perchloric acid to extract any remaining HMG proteins [17]. After 30 min at 0°C, the samples were centrifuged at 12 000 × *g* for 10 min. The supernatants were removed and dialyzed against SDS gel electrophoresis sample buffer. After electrophoresis, protein bands were visualized with the silver staining technique in [18]. To avoid decreasing the intensities of the stained bands, silver deposits on the surfaces of the gel were not removed.

3. Results

3.1. *NaCl dissociation of high molecular weight HMG proteins from purified double-stranded DNA*

A sample containing chicken erythrocytes HMG-1, HMG-2 and HMG-E, obtained as in section 2, was dialyzed against 0.05 M NaCl/1 mM Tris–HCl (pH 7.5) and applied to a double-stranded DNA-cellulose column that had been equilibrated to the same solvent. Protein peaks were found in the 0.05 M NaCl eluate and after application of 0.11 M NaCl and 0.2 M NaCl (fig.1). SDS–polyacrylamide gel electrophoresis

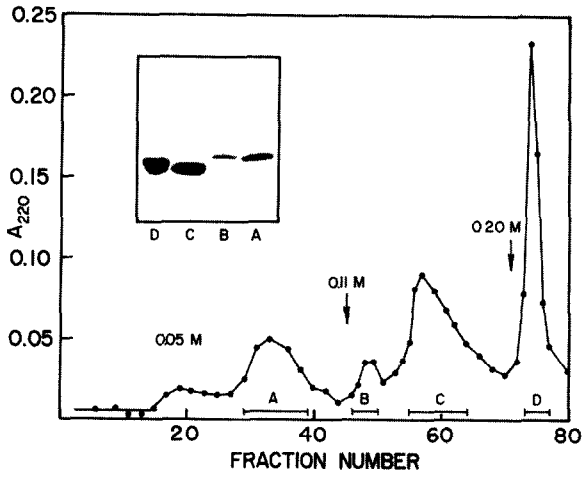

Fig.1. Chromatography of chicken erythrocyte HMG-1, HMG-2 and HMG-E on double-stranded DNA cellulose. The sample, ~2 mg protein, was applied in 0.05 M NaCl/1 mM Tris–HCl (pH 7.5). Later, solutions of 0.11 M NaCl/1 mM Tris–HCl and 0.20 M NaCl/1 mM Tris–HCl were applied to the column; the arrows indicate the points at which those solutions first appeared in the effluent stream. The column contained ~5 mg DNA and had dimensions of 2 × 35 cm. Flowrate: 15 ml/h. Fraction volume: 6.7 ml. HMG-1 has the lowest mobility in SDS–polyacrylamide gel electrophoresis of the high molecular weight HMG proteins, and HMG-E has the highest mobility of the 3 proteins [4,12]. Thus, from the inset, one can see that fractions A and B contain HMG-1, C contains HMG-E, and D contains predominantly HMG-2 and a small amount of HMG-E.

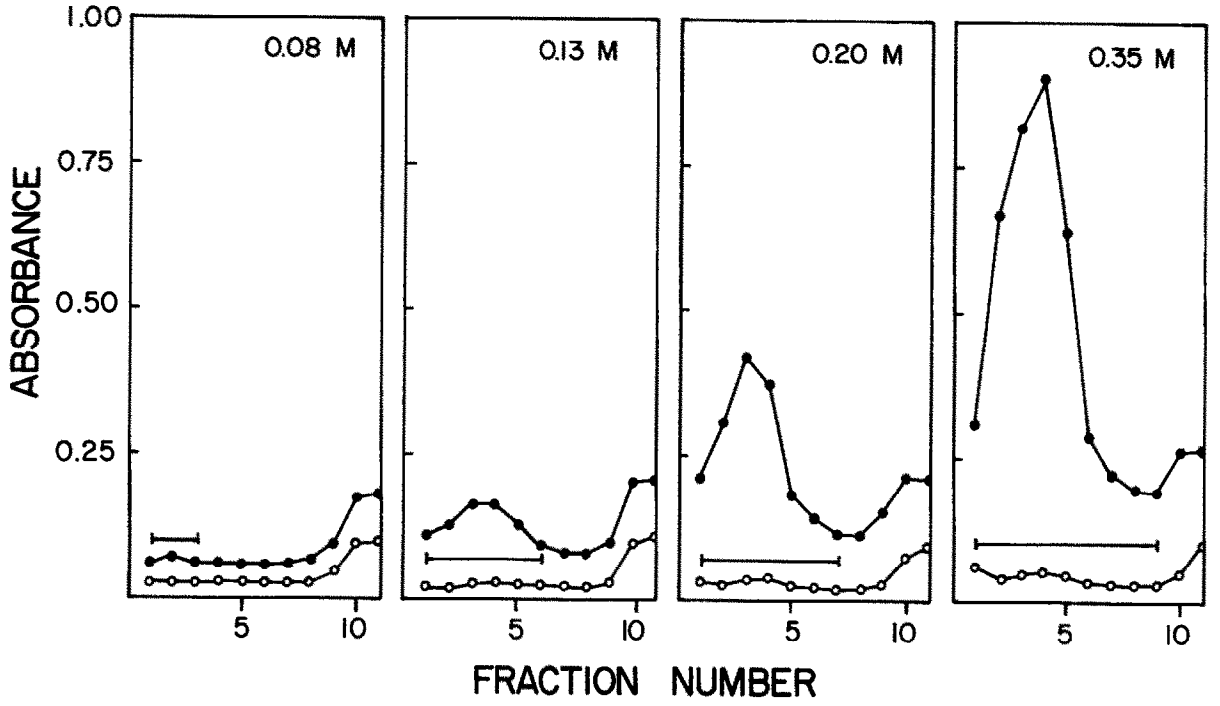

Fig.2. NaCl dissociation of proteins from chicken erythrocyte chromatin: A_{220} (•); A_{260} (○). Chromatin samples were adjusted to the indicated NaCl levels and centrifuged through 20% sucrose/1 mM Tris–HCl (pH 7.5) containing the same NaCl levels. Fractions were collected from the top of each tube. The rise in absorbance at fraction 9 of each tube is due to the 60% sucrose with which the tubes were pumped out. That sucrose had A_{220} 0.21 and A_{260} 0.11.

(fig.1 inset) showed that the major portion of HMG-1 eluted in 0.05 M NaCl. A small additional amount of HMG-1 eluted in 0.11 M NaCl, as did HMG-E. HMG-2 was eluted with 0.2 M NaCl.

3.2. *NaCl dissociation from chicken erythrocyte chromatin of the high molecular weight protein*

Centrifugation through NaCl-containing sucrose solutions efficiently separates NaCl-dissociated chromatin proteins from DNA and proteins that remain bound to DNA [16]. We used that approach to examine the dissociation of HMG-1, HMG-2 and HMG-E from chicken erythrocyte chromatin at selected NaCl levels. Spectrophotometric measurement of fractions recovered after centrifugations at 0.08, 0.13, 0.20 and 0.35 M NaCl are shown in fig.2. All of the DNA in each tube (64 A_{260} units) was in the pellet formed during centrifugation. (The absorbances of fractions 9–11 from each tube are accounted for by the absorbances at 220 and 260 nm of the 60% sucrose used to pump out the tubes' contents.) Therefore, the peaks of absorbance at the lower numbered fractions of each tube provide a measure of the protein released from chromatin at each NaCl level.

SDS–polyacrylamide gel electrophoresis (fig.3) showed that HMG proteins are the main components released ≤0.35 M NaCl, although at 0.35 M NaCl two histone H1 bands are also present in significant amounts. Since the HMG proteins predominate in the NaCl-dissociated proteins, the spectrophotometric measurements in fig.2 indicate that increasing amounts of HMG proteins are released from chromatin as NaCl is increased up to 0.35 M. That is confirmed by the relative intensities of HMG protein bands in tracks 1–4 of fig.3. (Note that the samples for the 4 tracks were derived from different amounts of chromatin, as described in the legend to fig.3.)

Two main points should be emphasized for comparison with the dissociation of the HMG proteins from double-stranded DNA.

(i) Only a very small portion of HMG-1 is released from chicken erythrocyte chromatin at 0.08 M NaCl. (Compare the intensity of the HMG-1 band in track 1 with the intensity of the HMG-1 band

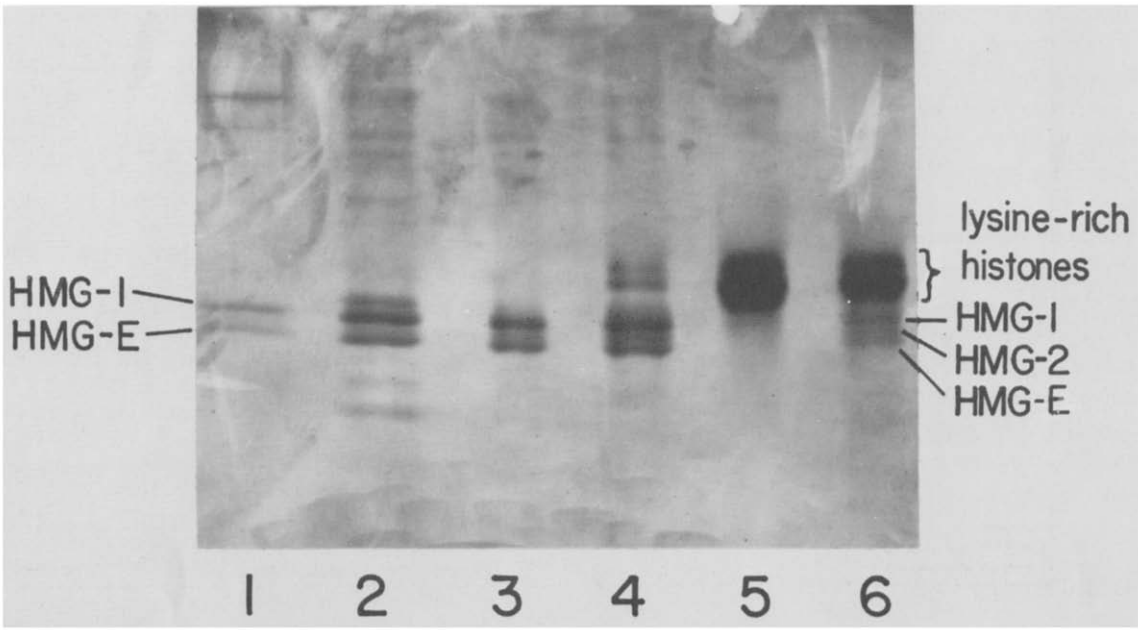

Fig.3. Polyacrylamide gel electrophoresis of chicken erythrocyte chromatin proteins. The samples for tracks 1–4 were proteins dissociated in the sucrose centrifugation experiment shown in fig.2. The NaCl level used to generate each sample and the amount of chromatin from which each gel sample was derived were: (1) 0.08 M NaCl, 320 μg chromatin DNA; (2) 0.13 M NaCl, 50 μg chromatin DNA; (3) 0.20 M NaCl, 3 μg chromatin DNA; (4) 0.35 M NaCl, 2 μg chromatin DNA. The samples for tracks 5 and 6 were perchloric acid extracts of pellets formed during centrifugation of chromatin in 0.35 M NaCl and 0.20 M NaCl, respectively. The samples were derived from 0.4 μg (5) and 0.3 μg (6) of chromatin DNA.

in track 4, the sample for which was derived from <1% as much chromatin as the sample for track 1.)

(ii) None of the high molecular weight HMG proteins was completely released from chicken erythrocyte chromatin by 0.20 M NaCl (cf. tracks 5 and 6).

4. Discussion

We have observed marked differences in the NaCl-promoted dissociations of high molecular weight HMG proteins from double-stranded DNA and from chromatin. Especially clear are the following differences: 0.08 M NaCl, a level sufficient to elute HMG-1 from columns containing double-stranded DNA, removed only a very small portion of HMG-1 from chromatin; and 0.2 M NaCl, a level sufficient to remove all of the high molecular weight HMG proteins from double-stranded DNA, did not completely remove those proteins from chromatin. We estimate that ≥50% of the HMG-1, HMG-2 and HMG-E found in chromatin isolated in 1 mM Tris–HCl remains bound to chromatin at 0.20 M NaCl.

We know from [12] that >0.2 M NaCl is necessary to remove HMG-1, HMG-2 and HMG-E from single-stranded DNA. (A significant portion of HMG-1 is removed with 0.30 M NaCl [12]; HMG-2 and the remainder of HMG-1 can be removed with <0.4 M NaCl (unpublished). In [12], 0.5 M NaCl was used to elute the high molecular weight HMG proteins in a sharp peak, not in an attempt to determine the lowest NaCl level at which the proteins could be eluted.) An explanation of these results may be that at least a major portion of each of those proteins is bound to single-stranded DNA regions in chromatin. Alternately, the proteins could bind more tightly to the negatively supercoiled form of DNA that exists in chromatin than to the non-supercoiled double-stranded DNA that we have immobilized on cellulose, or the proteins may bind not only to DNA in chromatin but also to proteins. Some support for the first possibility is provided by [19], where a population of mouse myeloma nucleosomes was isolated that apparently

contained 35–40% single-stranded DNA and stoichiometric amounts of HMG-1 and HMG-2.

Up to several % of the DNA purified from cells of higher vertebrates has been shown to be sensitive to digestion by the single-stranded DNA-specific S_1 nuclease from *Aspergillus oryzae* and is, therefore, presumed to be single-stranded [20,21]. However, in a microcomplement fixation study with antibodies against single-stranded DNA [22], <0.01% of the DNA in rabbit liver chromatin was accessible to those antibodies. Thus, regions of single-stranded DNA in chromatin might be complexed with proteins that prevent interaction of the antibodies with the single-stranded DNA. We suggest that the high molecular weight HMG proteins are plausible candidates for such proteins.

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References

[1] Goodwin, G. H., Sanders, C. and Johns, E. W. (1973) Eur. J. Biochem. 38, 14–19.
[2] Sterner, R., Boffa, L. C. and Vidali, G. (1978) J. Biol. Chem. 253, 3830–3836.
[3] Romani, M., Rodman, T. C., Vidali, G. and Bustin, M. (1979) J. Biol. Chem. 254, 2918–2922.
[4] Mathew, C. G. P., Goodwin, G. H., Gooderham, K., Walker, J. M. and Johns, E. W. (1979) Biochem. Biophys. Res. Commun. 87, 1243–1251.
[5] Watson, D. C., Peters, E. H. and Dixon, G. H. (1977) Eur. J. Biochem. 74, 53–60.
[6] Walker, J. M., Goodwin, G. H. and Johns, E. W. (1979) FEBS Lett. 100, 394–398.
[7] Walker, J. M., Hastings, J. R. B. and Johns, E. W. (1977) Eur. J. Biochem. 76, 461–468.
[8] Rabbani, A., Goodwin, G. H. and Johns, E. W. (1978) Biochem. J. 173, 497–505.
[9] Rabbani, A., Goodwin, G. H. and Johns, E. W. (1978) Biochem. Biophys. Res. Commun. 81, 351–358.
[10] Watson, D. C., Wong, N. C. W. and Dixon, G. H. (1979) Eur. J. Biochem. 95, 193–202.
[11] Bidney, D. L. and Reeck, G. R. (1978) Biochem. Biophys. Res. Commun. 85, 1211–1218.
[12] Isackson, P. J., Fishback, J. L., Bidney, D. L. and Reeck, G. R. (1979) J. Biol. Chem. 254, 5569–5572.
[13] Bidney, D. L. and Reeck, G. R. (1977) Biochemistry 16, 1844–1849.
[14] Alberts, B. and Herrick, G. (1971) Methods Enzymol. 21, 198–217.
[15] Laemmli, U. K. (1970) Nature 227, 680–685.
[16] Bidney, D. L. and Reeck, G. R. (1978) Biochim. Biophys. Acta 521, 753–761.
[17] Goodwin, G. H., Walker, J. M. and Johns, E. W. (1978) Biochim. Biophys. Acta 519, 233–242.
[18] Switzer, R. C. iii, Merril, C. R. and Shifrin, S. (1979) Anal. Biochem. 98, 231–237.
[19] Jackson, J. B., Pollock, J. M. jr and Rill, R. L. (1979) Biochemistry 18, 3739–3748.
[20] Leibovitch, S. A. and Harel, J. (1978) Nucleic Acids Res. 5, 777–787.
[21] Collins, J. M., Berry, D. E. and Cobbs, C. S. (1977) Biochemistry 16, 5438–5444.
[22] Levy, S. and Simpson, R. T. (1973) Nature 241, 139–141.