ISOLATION AND SEPARATION OF CHICKEN ERYTHROCYTE HIGH MOBILITY GROUP NON-HISTONE CHROMATIN PROTEINS BY CHROMATOGRAPHY ON PHOSPHOCELLULOSE

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1. Introduction

The high mobility group (HMG) proteins are well characterized non-histone chromatin proteins that were first isolated from calf thymus in [1]. The proteins can be extracted from calf thymus chromatin with 0.35 M NaCl, are soluble in 2% trichloroacetic acid, have relatively high mobilities in acid/urea–polyacrylamide gel electrophoresis, and contain high levels of both acid and basic amino acid residues [2]. Their distinctive physiochemical properties allow the HMG proteins to be readily identified (at least in higher organisms) and have in large part been responsible for the proteins' having been studied in many laboratories. In chicken erythrocytes, the HMG proteins are HMG-1, HMG-2, HMG-E, HMG-14, and HMG-17 [3,4]. We have shown that HMG-1, HMG-2, and HMG-E (sequence homologs [5], which we refer to as the high molecular weight HMG proteins) all have the distinctive property of binding selectively to single-stranded DNA at roughly physiological ionic strength [6]. Red cell HMG-14 and HMG-17 are associated preferentially with nucleosomes containing transcribed DNA sequences [7]. All of the chicken erythrocyte HMG proteins are thus likely to be the subjects of continued, intensive investigation.

The principal large-scale procedures for purification of individual HMG proteins have been developed [8,9]. We report here the use of phosphocellulose chromatography as the first and most important step in an alternate purification method that has distinct advantages over existing procedures.

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2. Methods

Nuclei and chromatin were prepared from chicken erythrocytes (Pel Freez) as detailed in [10]. Nuclei were isolated by repeatedly homogenizing erythrocytes in 1% Triton X-100/0.25 M sucrose/3 mM $CaCl_2$/50 mM Tris–HCl (pH 7.5). Chromatin was obtained by homogenizing the nuclei in 1 mM Tris–HCl (pH 7.5). The swollen gel of unsheared chromatin (1100 ml at 2 mg DNA/ml) was adjusted to 0.35 M NaCl by adding solid NaCl. After being stirred for 30 min at 4°C the sample was centrifuged for 30 min at 25 000 × g at 4°C. The supernatant was used as the 0.35 M NaCl extract of chromatin. All buffers used in the purification of nuclei and chromatin and in obtaining the 0.35 M NaCl extract included 0.1 mM phenylmethane sulfonyl fluoride. Omitting that reagent led to preferential loss of HMG-14 and HMG-17.

Whatman P-11 phosphocellulose (lot 2111341) was treated with NaOH and HCl as recommended by the manufacturer, equilibrated to 0.35 M NaCl/10 mM Tris–HCl (pH 7.5), and packed in a 2 × 100 cm column under 10 lb/in^2 of nitrogen.

The 0.35 M NaCl extract (1100 ml) was applied directly to the phosphocellulose column. After the sample had been applied, the column was washed with 0.35 M NaCl/10 mM Tris–HCl (pH 7.5) to remove unbound material. The HMG proteins were then eluted by successive applications of 0.450 M NaCl, 0.465 M NaCl, and 0.55 M NaCl, all in 10 mM Tris–HCl (pH 7.5). A peak that contained principally histone H1 was eluted with 2 M NaCl/10 mM Tris–HCl. The chromatography was carried out at 4°C at a flow rate of 60 ml/h.

Additional purification was necessary for several of the HMG proteins. HMG-1 and HMG-2 were each separated from HMG-14a by chromatography on carboxymethyl-Sephadex C-25 at pH 9, as in [9]. Contaminants of HMG-14b were removed by diluting that portion of the phosphocellulose effluent that contained HMG-14b to 0.2 M NaCl and applying the material to a carboxymethyl-Sephadex C-25 column equilibrated to 0.2 M NaCl/10 mM Tris–HCl (pH 7.5). The contaminants were not retained by the column, whereas HMG-14b was. It was subsequently eluted with 0.5 M NaCl/10 mM Tris–HCl.

We routinely concentrate HMG protein samples from column effluents by dilution, application to phosphocellulose, and elution with a high [NaCl]. For instance, HMG-E from phosphocellulose chromatography is diluted to 0.35 M NaCl and applied to a 1 × 30 cm column of phosphocellulose equilibrated to 0.35 M NaCl/10 mM Tris–HCl. The HMG-E is then eluted in a concentrated peak by applying 0.7 M NaCl/10 mM Tris–HCl. The high M_r HMG proteins and HMG-14 and HMG-17 are dialyzed in Spectrapor tubing with nominal M_r cutoffs of 6000–8000 and 3500, respectively.

Acid/urea–polyacrylamide gel electrophoresis in 20% gels was done as in [11]. SDS–polyacrylamide electrophoresis was done as in [12] in gels polymerized from 15% acrylamide/0.2% bis-acrylamide. Protein samples were prepared for electrophoresis by precipitating the protein with 25% trichloroacetic acid and washing the precipitates with acetone. Proteins were hydrolyzed in evacuated tubes for amino acid analysis for 24 h at 120°C in 5.7 N HCl. Amino acid analyses were performed on a Dionex microbore column with a ninhydrin detection system and a Columbia Scientific Supergrator III integrator.

Protein concentrations were estimated with the Coomassie blue binding assay in [13] with ovalbumin (Sigma A5503) as standard. By comparison with concentrations estimated from the proteins' extinction coefficients at 280 nm, we believe that the Coomassie blue binding assay with ovalbumin as standard gives estimates for HMG-1, HMG-2, and HMG-E that are ~100% too high. We do not know how accurate that method is for HMG-14 or HMG-17 or other proteins in the 0.35 M NaCl extract of erythrocyte chromatin. We report values for all proteins determined by the Bradford method [13] for the sake of internal consistency.

3. Results

All of the chicken erythrocyte HMG proteins are retained when a 0.35 M NaCl extract of chromatin is applied directly to a phosphocellulose column equilibrated to 0.35 M NaCl/1 mM Tris–HCl (pH 7.5). A phosphocellulose column chromatogram is shown in fig.1. Acid/urea–polyacrylamide gel electrophoretic analysis of portions of the effluent are shown in fig.2. We examined the same samples by SDS–polyacrylamide gel electrophoresis to distinguish HMG-2 and HMG-E (not shown). The flowthrough (not included in the elution profile in fig.1) had a high absorbance due in part to the phenylmethane sulfonylfluoride in the extract. The flowthrough contained 25% of the protein applied to the column, as estimated by the Coomassie blue binding assay [13]. Applying 0.450 M NaCl resulted in elution of several peaks. The size of the first (fractions 42–55) varied in apparent correspondence with the purity of the nuclei, and it contained several proteins. A shoulder on the trailing edge of that peak (fractions 56–75) contained a protein with electrophoretic mobilities of HMG-E. Continued application of 0.450 M NaCl resulted in elution of a large, asymmetric peak (fractions 113–160) that contained the major portion of HMG-E. A preliminary examination by isoelectric focusing has not revealed any differences in the HMG-E from three portions of that peak, and it may be that the apparent heterogeneity indicated by the shape of the peak is a chromatographic artifact. The shoulder on the leading edge of the HMG-E (fractions 97–112) contains some HMG-E and histone H5, a small amount of which is removed from chicken erythrocyte chromatin with 0.35 M NaCl.

HMG-1 (fractions 190–250) and HMG-2 (fractions 250–310) were eluted with 0.465 M NaCl, as was a species of HMG-14 that we call HMG-14a. Electrophoretic analysis of individual fractions (not shown) revealed that HMG-14a eluted as a single peak with a maximum at about fraction 240.

A second species of HMG-14 (that we call HMG-14b) was eluted by applying 0.55 M NaCl (fractions 365–374), along with some other proteins, apparently including small amounts of HMG-1 and HMG-2. Continued application of 0.55 M NaCl resulted in elution of HMG-17 (fractions 380–400) in a nearly homogeneous state.

HMG-1, HMG-2, HMG-14a, and HMG-14b were

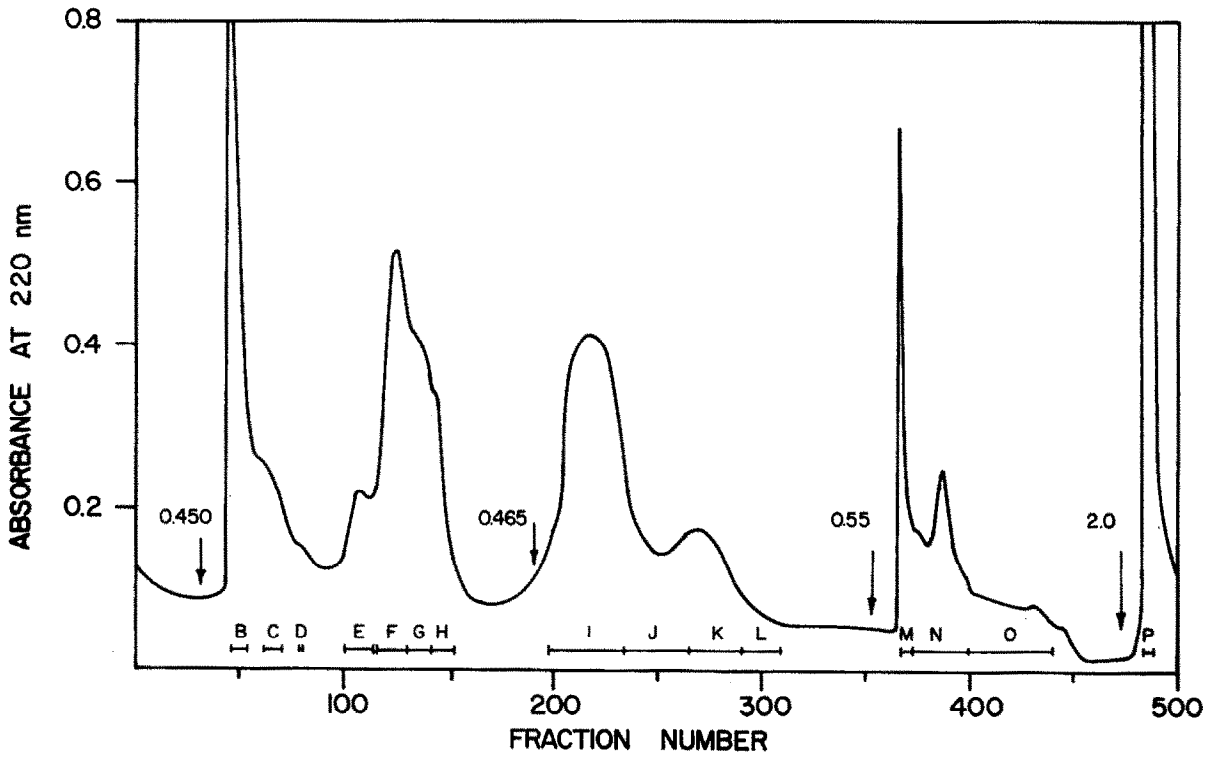

Fig.1. Chromatography of salt-extracted chicken erythrocyte chromatin proteins on phosphocellulose. The sample was a 0.35 M NaCl extract of chromatin. Chromatography was done as in section 2. The flowthrough is not shown. The arrows indicate points at which solutions of 0.450 M, 0.465 M, 0.55 M, and 2.0 M NaCl were applied. The bars indicate fractions combined for analysis by gel electrophoresis (see fig.2).

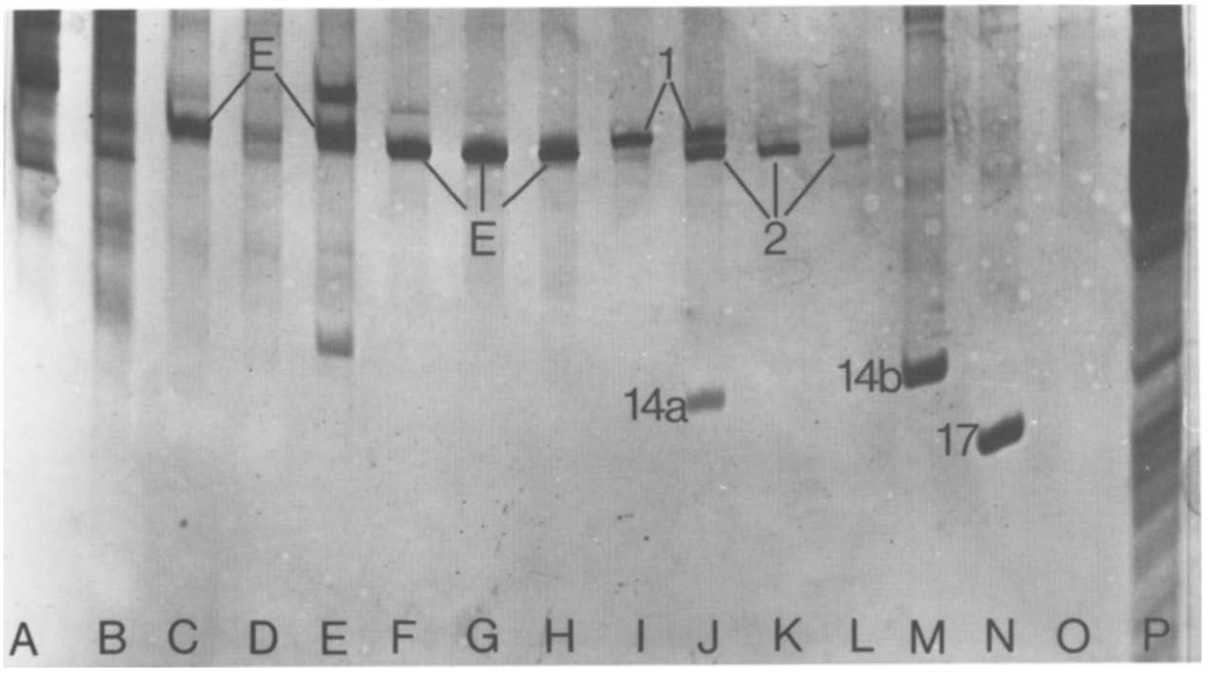

Fig.2. Acid/urea–polyacrylamide gel electrophoresis of combined fractions from phosphocellulose chromatography of chicken erythrocyte chromatin proteins. The sample for (A) was from the breakthrough of the phosphocellulose column. The samples for (B–P) were from the corresponding combined fractions indicated by bars in fig.1. In several of the tracks, HMG proteins are identified by appropriate labels.

further purified as in section 2. SDS– and acid/urea–polyacrylamide gel electrophoresis analyses of the purified proteins are shown in fig.3,4. Using the Coomassie blue binding assay [13] with ovalbumin as standard, we estimated yields from chromatin containing 2.2 g DNA to be: 23 mg HMG-1; 6 mg HMG-2; 28 mg HMG-E; 3 mg HMG-14b; and 4 mg HMG-17. As discussed in section 2, we believe the estimates for the high M_r HMG proteins to be too high.

Amino acid compositions of the purified HMG proteins are given in table 1. The compositions agree reasonably well with those in [4]. The amino acid compositions of HMG-14a and HMG-14b are of particular interest; we believe they are sufficiently different to suggest that the two proteins have different, but presumably very similar, primary structures. The most reliable indicators of significant differences are the relative amounts of Glu, Gly, and Ala, all of which are abundant amino acids in the proteins, stable to acid hydrolysis, and readily quantified. The two proteins have different Gly/Ala ratios and different Glu/Ala ratios. The differences in those ratios have been observed in two preparations of each protein and they thus seem unlikely to have resulted from contamination, either by proteins or by free amino acids. We suggest, therefore, that chicken erythrocytes contain two distinct HMG-14 species. In [9] an HMG-14 peak from carboxymethyl-Sephadex chromatography was divided into two parts and it was stated that materials from both parts had identical amino acid compositions.

We have also chromatographed chicken erythrocyte HMG proteins on phosphocellulose column buffered with 10 mM sodium phosphate (pH 7.5) rather than with Tris–HCl [14]. In that system, HMG-1 and HMG-2 are better separated from each other, but HMG-14a elutes with HMG-E, HMG-14b elutes with HMG-1, and HMG-17 elutes with HMG-2. Thus, for chicken erythrocyte proteins, we prefer the Tris–HCl-buffered system. It might well be, however, that phosphate buffer will be preferable for the chromatography of HMG proteins from other sources on phosphocellulose.

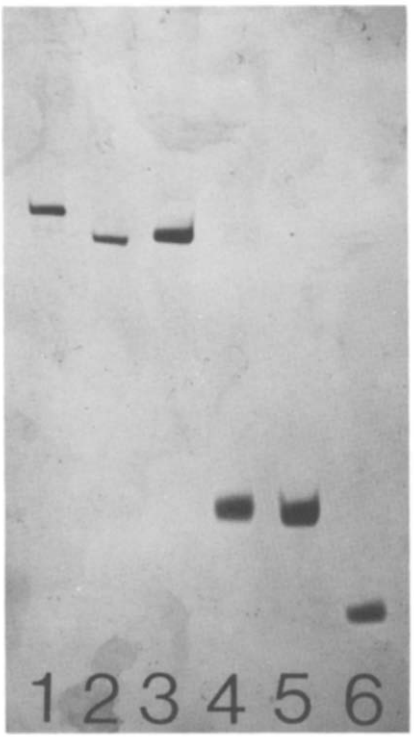

Fig.4. Acid/urea–polyacrylamide gel electrophoresis of purified HMG proteins: (1) HMG-1; (2– HMG-2; (3) HMG-E; (4) HMG-14a; (5) HMG-14b; (6) HMG-17.

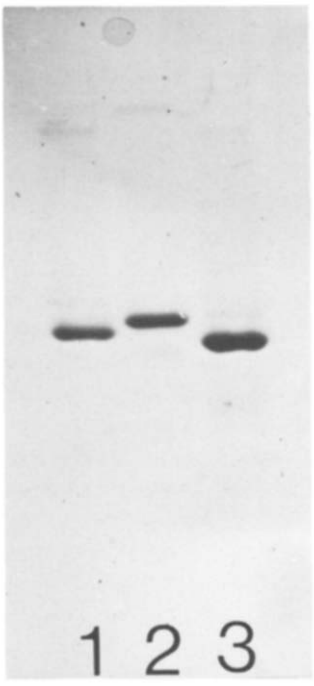

Fig.3. SDS–polyacrylamide gel electrophoresis of purified high molecular weight HMG proteins: (1) HMG-2; (2) HMG-1; (3) HMG-E.

Table 1
Amino acid compositions of purified chicken erythrocyte HMG proteins[a]

	HMG-1	HMG-2	HMG-E	HMG-14a	HMG-14b	HMG-17
Asp	12	8.9	13	7.5	9.4	9.9
Thr	2.4	2.1	2.3	2.9	2.7	2.4
Ser	4.3	5.5	3.9	3.6	1.9	2.7
Glu	15	17	12	16	13	10
Pro	6.5	7.8	6.3	14	15	11
Gly	6.8	8.5	8.7	9.0	6.4	12
Ala	9.3	10	12	20	22	18
Val	3.6	2.0	4.2	1.2	0.6	2.9
Met	2.0	1.8	1.7	0	0	Trace
Ile	1.7	1.6	1.9	0	0	0.5
Leu	3.1	2.6	2.8	2.3	1.5	2.0
Tyr	2.7	2.5	2.4	0	0	Trace
Phe	5.2	3.4	5.0	0	0	Trace
His	2.9	3.1	1.6	0.7	1.5	0.7
Lys	18	17	20	23	24	23
Arg	5.1	5.9	4.4	1.5	1.6	4.4

[a] All values are in mol %. No corrections have been made for hydrolytic losses

The compositions of all the proteins except the HMG-14 species are averages of single analyses performed on two preparations of each. The compositions of HMG-14a and HMG-14b are from single analyses of single preparations of each protein. The baseline in the Arg region of the chromatograms was complex, possibly because of low levels of ammonia in the eluent buffers. As a result, the Arg values for HMG-14a and HMG-14b (the analyses of which were performed at low sample levels) probably are too low

4. Discussion

We believe that phosphocellulose chromatography offers significant advantages over currently available schemes for large scale purification of individual HMG proteins. The procedures in [8,9] employ conditions and solvents (such as trichloroacetic acid and acetone) that are well-recognized protein denaturants [15,16]. That approach to purification is entirely appropriate for isolation of proteins for covalent structure determination (a major interest of those investigators), but is less well-suited to studies based on the presumption that the isolated proteins exist in their native states. The ability of preparations of HMG-14 and HMG-17 that have been exposed to high concentrations of trichloroacetic acid to specifically reconstitute the DNase I sensitivity of transcribed genes [17,18] is a convincing demonstration that at least a portion of the molecules in such preparations can, in the presence of chromatin, return to or adopt their native state. For the high M_r HMG proteins no such assay is currently available. The rather high content of α-helix in calf thymus HMG-1 and HMG-2 prepared as in [8,9] is an indication that the proteins exist in highly folded states [19]. It is not uncommon, however, for small changes in conformation to greatly alter biological activity of proteins, and it is not impossible for proteins in highly folded states to be biologically inactive. In the absence of a functional assay to monitor a return to the native state, the safest course of action is to avoid overt denaturing conditions if possible (detailed in [20]). Phosphocellulose chromatography or sequential DNA chromatography [6] allows purification of individual HMG proteins without exposure to such conditions. We have been unable to prepare double-stranded DNA columns of high capacity that are free of single-stranded DNA, so we prefer phosphocellulose chromatography for large scale purifications. The high M_r HMG proteins purified by either phosphocellulose chromatography or sequential DNA chromatography have exhibited no tendency to precipitate even at low ionic strengths. We have, for instance, dialyzed a 0.5 mg/ml solution of HMG-1 against 1 mM Tris–HCl (pH 7.5) with no indication of precipitation. That contrasts with the behavior of

high M_r HMG proteins purified as in [8], at least in our hands.

We have not yet systematically explored chromatography of HMG proteins from sources other than chicken erythrocytes on phosphocellulose. We have found, however, that purified rat HMG-1 and HMG-2 bind to phosphocellulose similarly to their chicken homologs, so we suspect that phosphocellulose chromatography will be a powerful tool in isolating and fractionating HMG proteins from a wide range of organisms.

Acknowledgements

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