A new procedure for purifying histone pairs H2A + H2B and H3 + H4 from chromatin using hydroxylapatite

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ABSTRACT

A method to purify histone groups H2A+H2B and H3+H4 using dissociation with NaCl and hydroxylapatite chromatography is presented. The procedure is simple, involves mild solvents, and provides milligram quantities of histones of high purity. The histone pairs prepared by this method can regenerate chromatin-like characteristics when combined and reconstituted with DNA.

INTRODUCTION

Recent investigations into the internal structure of the nucleosome of chromatin have indicated that of the four core histones, H2A, H2B, H3 and H4, the arginine rich group H3+H4 have a central role in organizing the DNA[1-6]. Further studies of this interaction and the function of the slightly lysine-rich group, H2A+H2B, would be facilitated if a simple method for purifying milligram quantities of the histone groups was available.

At present a frequently used purification scheme is that introduced by Van der Westhuyzen and Von Holt[7]. It involves an initial extraction of the histones from the DNA of chromatin by dissociating them with protamine. The histones must then be concentrated and the protamine removed by gel filtration chromatography. The histone groups are next fractionated at pH 5.0 by gel filtration chromatography into two peaks, one containing H2A and H2B, and the other H3, H4, and H1. The H3+H4 is separated from H1 by precipitation in 70% ammonium sulfate.

Other methods are available for fractionating histones into groups. Treatment of chromatin with 2 M NaCl quantitatively separates all of the histones from DNA[8]. Exposure of chromatin to lower NaCl concentrations can selectively strip off specific groups of histones and these histones can then be recovered from the remaining nucleoprotein by centrifugation or gel filtra-tion chromatography[9-11]. Such methods have not been extensively used to prepare H2A+H2B and H3+H4, and in our hands the purified fractions are fre-

quently contaminated with other histone species.

Hydroxylapatite was first used to fractionate histones by Faulhaber and Bernardi[12]. They equilibrated chromatin with solvents containing 0.7 mM potassium phosphate, pH 6.8, and various concentrations of KCl, and subjected it to chromatography on hydroxylapatite. The initial conditions were such that both the DNA and histones were retained on the column. The column was next eluted with the same KCl concentration and a linear potassium phosphate gradient from 0.7 mM to 500 mM. In experiments run at low KCl concentrations, the histones remained bound to the DNA and the chromatin eluted in a single nucleo-protein peak. However if the chromatography was performed at progressively higher KCl concentrations, more and more free protein was found in a new peak eluting prior to that containing the DNA. When 3 M KCl was used, all the protein was present in this peak, and the DNA, now protein free, eluted later in the potassium phosphate gradient. The amino acid profile of the protein peak was found to be dependent upon the KCl concentration used in the experi-ment. In particular, comparison of the lysine to arginine ratios indicated that the lysine abundant histones could be selectively eluted at lower KCl concentrations than the arginine abundant species.

In this paper, a procedure is presented that combines the NaCl dissocia-tion and hydroxylapatite methods described above. The hydroxylapatite column is used to support the DNA component of chromatin while increasing NaCl con-centrations are used to strip off sequentually the H1+H5, H2A+H2B, and H3+H4 histone groups. This method has the advantage of requiring only a single chromatography step that both separates the histones from the DNA and fraction-ates them into the desired groups. The histones prepared in this way show no signs of degradation and are capable of regenerating the folded nucleosome structure when reconstituted onto DNA[13]. In a recently published study, Bloom and Anderson[14] described a somewhat similar fractionation on hydroxylapatite using solvents containing urea.

MATERIALS AND METHODS

Chromatin was prepared from Triton X-100 washed duck or chicken erythrocyte nuclei[15] and resuspended after the final centrifugation in 1 mM Tris-HCl, pH 8.0, 0.1 mM Na_2EDTA. The chromatin was sheared for 90 sec at 90 V in a Virtis homogenizer and then centrifuged at 16,000 x g for 30 min in an HB-4 Sorvall rotor at 4°C to remove any insoluble material.

The sheared chromatin was fractionated on a hydroxylapatite (Bio-gel HTP) column as described below. Column effluent was monitored spectrophotometri-

cally at 230 and 260 nm. The peak fractions were pooled and concentrated in an Amicon ultrafiltration apparatus containing a PM-10 membrane. The histones in the various fractions were identified by their electrophoretic migration on SDS polyacrylamide slab gels[1] or on Triton X-100, acid-urea polyacrylamide slab gels using a modification (R. D. Camerini-Otero, in preparation) of the procedure of Alfageme et al.[16]. The gels were stained with Coomassie blue, photographed, and the negatives scanned on a Joyce-Loebl microdensitometer. Spectrapor 1 tubing (Spectrum Medical Industries, Inc.) was used in all dialysis.

RESULTS

Sheared chromatin binds to hydroxylapatite in 0.1 M potassium phosphate, pH 6.7 and the DNA component remains bound at NaCl concentrations up to 2 M. Using hydroxylapatite as a support for the DNA of chromatin, the histone groups (lysine-rich H1 and H5, slightly lysine-rich H2A and H2B, and arginine-rich H3 and H4) can be stripped sequentually from chromatin by dissociating each group at increasing NaCl concentrations. The following procedure was developed to maximize the yield of each histone group and minimize the cross contamination of the H2A+H2B and H3+H4 fractions.

The sheared chromatin at approximately 1 mg/ml DNA was dialyzed overnight at 4°C against 0.63 M NaCl, 0.1 M potassium phosphate, pH 6.7. During the dialysis, the nucleoprotein precipitated and then went back into solution as the final NaCl concentration was reached. It was then centrifuged at 16,000 x g for 30 min in an HB-4 Sorvall rotor at 4°C to remove any insoluble material. An amount of chromatin containing 34 mg of DNA was applied to a 2.5 x 20 cm hydroxylapatite column that had been equilibrated with the same buffer at 4°C. While collecting 18 ml fractions, the column was washed with 400 ml of the starting buffer and the absorbance of fractions measured at 230 and 260 nm. During the initial wash, two poorly resolved 230 nm peaks were eluted (Fig. 1). SDS polyacrylamide gel electrophoresis (Fig. 2) of the two peaks identified the first (peak 1) as H1 and non-histone proteins. Peak 2 was predominantly H5 with a moderate contaminant of H2A+H2B. The trailing edge of peak 2 contained mostly H2A+H2B.

The running buffer was next stepped to 0.93 M NaCl, 0.1 M potassium phosphate, pH 6.7, and 350 ml was washed through the column. A single 230 nm peak (peak 3) with a trailing edge was eluted. The SDS gel electrophoresis pattern indicated that it consisted of H2A+H2B.

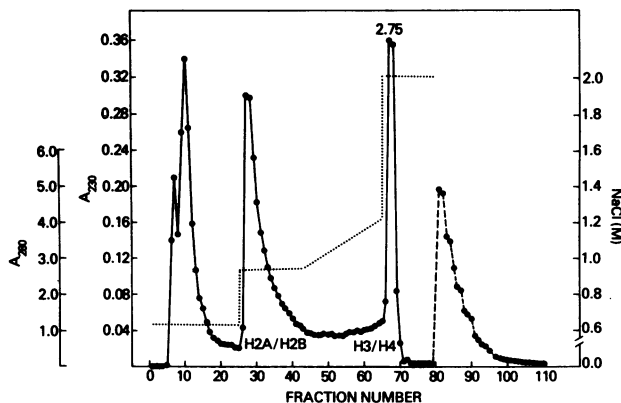Next a 500 ml linear NaCl gradient from 0.93 M to 1.20 M NaCl was run

Fig. 1. Hydroxylapatite column chromatography. Chromatin containing 34 mg of DNA in 0.63 M NaCl, 0.1 M potassium phosphate, pH 6.7, was loaded onto a 2.5 x 20 cm column, and eluted in 18 ml fractions at 60 ml/hour. The NaCl concentration of the running buffer is indicated by the dotted line. The concentration of potassium phosphate (pH 6.7) was maintained at 0.1 M until tube 79, then stepped to 0.5 M. The optical absorbance of the fractions was determined at 230 nm (solid line) and 260 nm (dashed line). Purified H2A+H2B and H3+H4 were obtained by pooling fractions 26-42 and 66-69 respectively

through the column while maintaining the potassium phosphate at 0.1 M. SDS gels run on samples taken from the eluted 230 nm valley (between peaks 3 and 4) showed that both H2A+H2B and H3+H4 were present. This mixture of the two histone groups indicated that a portion of the H3+H4 began to elute from the column before all of the H2A+H2B was removed. If the initial step to 0.93 M NaCl was taken instead to a higher NaCl concentration in order to elute more of the H2A+H2B, it was found that H3+H4 began to elute and contaminate the H2A+H2B peak. Hence the gradient was used to wash the remaining H2A+H2B from the column yet minimize the removal of H3+H4 at this stage.

The bulk of the arginine-rich histones was next eluted from the column in a single, sharp peak (peak 4) with 200 ml of 2 M NaCl, 0.1 M potassium phosphate, pH 6.7. The buffer was changed to 0.5 M potassium phosphate, pH 6.7 and the DNA was removed from the column (peak 5). The NaCl was omitted from this wash to prevent precipitation of sodium phosphate at 4°C. The column was reused after removing the top 5 mm of hydroxylapatite and equilibrating again with starting buffer.

Samples from various fractions along the leading,.middle and trailing edge of the H2A+H2B and H3+H4 peaks were electrophoresed on SDS gels. The
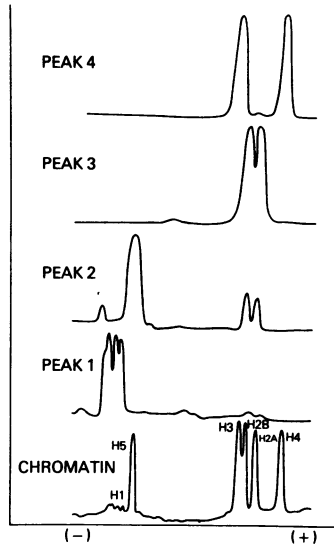
Fig. 2. Electrophoretic patterns of column fractions shown in Fig. 1. SDS polyacrylamide gel electrophoresis; gels were stained, photographed and traced as described in Methods. Migration is from left to right. Samples were taken from peak tube of peaks 1 through 4. A chromatin pattern is included at bottom for reference.

ratios of H2A to H2B and of H3 to H4 remained constant across the peaks.

The fractions indicated in the caption to Fig. 1 were pooled. This yielded 9.8 mg of H2A+H2B and 13.5 mg of H3+H4, or 66% and 95% respectively of the amount of H2A+H2B and H3+H4 loaded onto the column. The lost histones are accounted for in the discarded fractions between peaks.

Samples from the pooled fractions of H2A+H2B and H3+H4 were electrophoresed on an SDS-polyacrylamide slab gel (Fig. 3). The ratios of H2A to H2B and H3 to H4 were the same as those present in chromatin, indicating that no single histone was lost on the column. Because the H2B and H3 peaks were not totally resolved on this gel, another gel electrophoresis system was used to detect any cross contamination between fractionated pairs. Samples were electrophoresed on 12.5% polyacrylamide slab gels in Triton X-100, with acid-urea buffer (Fig. 4). Under these conditions histone H2B and H3 are well separated and the gel demonstrates minimal contamination of one pair with histones of the other.

A comparison of A_{230} to A_{260} for the protein peaks showed that each peak contained less than 1% DNA. SDS gels of samples from the DNA peak indicated
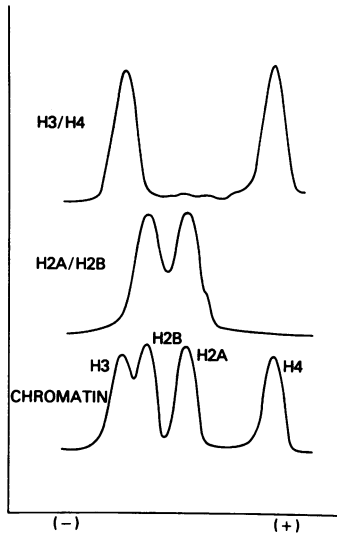
Fig. 3   SDS gel electrophoresis of samples from pooled H2A+H2B and H3+H4 peaks.
Migration is from left to right.   Tracings are taken from the region of gel
where histones migrated.   A chromatin pattern is included at bottom for
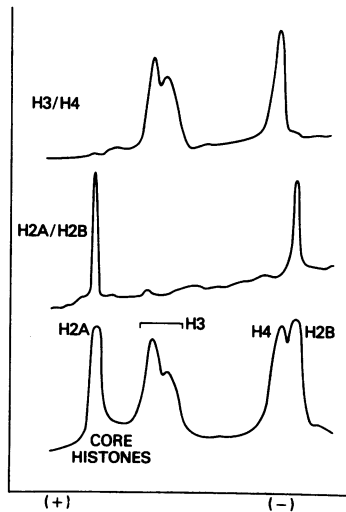comparison.

Fig. 4.   Triton X-100, acid-urea polyacrylamide gel electrophoresis of samples
from pooled H2A+H2B and H3+H4 peaks. Migration is from left to right.   A
chromatin pattern is included at bottom for reference.

that less than 1% of the protein loaded onto the column eluted with the DNA fraction.

Several methods were used to determine if the histones were altered by the extraction procedure. The arginine-rich pair was capable of inducing supercoils in closed, circular ColE1 DNA[4], and was able to protect discrete fragments of large DNA from staphylococcal nuclease digestion to an extent similar to histone H3+H4 prepared in other fashions[6]. When equimolar mixtures of H2A+H2B and H3+H4 were reconstituted (at a total protein:DNA ratio of 1.2 gr/gr) onto DNA of approximately nucleosome size (157 base pairs weight average), more than 2/3 of the product sedimented as an 11S particle. The histones are thus capable of compacting DNA into nucleosome-like particles.

DISCUSSION

At low phosphate concentrations, the DNA of chromatin is adequately exposed so that it can bind to hydroxylapatite[12]. Washing with up to 2 M NaCl does not release the DNA. The interaction of chromatin and hydroxylapatite does not alter the ability of increasing NaCl concentration to sequentually strip H1+H5, H2A+H2B, H3+H4 from the nucleoprotein. If the phosphate concentration is 0.1 M, as was used in this procedure, the histones released by salt stripping do not bind to the hydroxylapatite and can be eluted from the column.

In the present procedure, H1 and H5 are dissociated from the nucleoprotein in 0.63 M NaCl, 0.1 M potassium phosphate before the sample is loaded onto the hydroxylapatite. During the initial column wash, they are eluted as separate peaks; H5 is slightly retarded during its passage through the column. Because the aim of this procedure is to produce pure H2A+H2B and H3+H4 under the mildest possible conditions, no effort was made to separate H1 and H5 further or to recover them independently of the nonhistone proteins. Conditions can be chosen to accomplish this if desired[14].

The conditions used for the present purification are very mild. Extremes of pH and other conditions which might chemically modify proteins are avoided. Most methods of reconstituting nucleoproteins involve exposure to 2 M NaCl, and the present procedure uses conditions no harsher than this. The histones prepared by this technique are unaltered to the extent that they retain the ability to fold DNA, protect discrete fragments of DNA from staphylococcal nuclease digestion, and generate supercoils (see above).

The main benefit of this procedure is its simplicity. A single column chromatography is all that is required. It avoids repeated concentrating steps and gel filtration to remove reagents. The phosphate and salt are easily

dialyzed out of the protein peaks. The histone pair fractions have little or no cross contamination. Therefore this method should provide a ready source of arginine-rich and slightly lysine-rich histone pairs to facilitate further investigation of their role in chromatin structure.

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