Histones H2A and H2B are neighbors along DNA in chromatin: characterization of subnucleosomal particles containing H2A+H2B

Daniel A. Nelson, Darlene K. Oosterhof, and Randolph L. Rill*

Department of Chemistry and Institute of Molecular Biophysics, The Florida State University, Tallahassee, FL 32306, USA

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ABSTRACT

Two specific slow sedimenting nucleoprotein particles containing equimolar amounts of histones H2A and H2B and 38 or 49 base pair (bp) lengths of DNA have been isolated by centrifugation on sucrose gradients. The 3.4S particles containing 38 bp DNA and H2A+H2B thermally denature at 61°, considerably higher than Proteinase K treated particles (44°), but lower than 11S nucleosomes (76°). Treatment with Proteinase K increases the circular dichroism of 3.4S particles at 280 nm by 63% and decreases the sedimentation coefficient to 2.1S. These results indicate that H2A and H2B are proximate along DNA in nucleosomes and alone can alter the optical activity and perhaps conformation of local regions of DNA.

INTRODUCTION

Electron microscopy has shown that 100Å⁰ chromatin fibrils appear beaded, indicating that histones locally fold DNA into complexes representing fundamental units of chromatin structure, commonly termed "nucleosomes."¹⁻⁴ Monomeric and oligomeric nucleosomes can be isolated from chromatin after cleavage of interconnecting regions with staphylococcal nuclease.⁴⁻⁶ Analyses have shown that nucleosomes consist of a compact "core" containing two copies each of histones H2A, H2B, H3 and H4 associated with 140 base pairs (bp) of DNA;⁷⁻⁹ plus an extensible 40-60 bp "spacer" region covered by H1 histones.¹⁰⁻¹²

Staph. nuclease further fragments chromatin DNA into ten to twelve discrete species from 40 to 160 bp in length, differing by about 10 bp,¹³⁻¹⁴ arising from internal cleavages of the repeat units.¹⁴⁻¹⁶ Previous studies by Rill et al.¹⁷ and Weintraub¹⁵ suggested that discrete nucleoprotein fragments are generated by nuclease corresponding to each subnucleosomal DNA species. This report describes the isolation and characterization of subnucleosomal particles containing a specific pair of

histones, H2A+H2B, associated with ~40-50 bp length DNA, providing direct evidence that the major binding sites of these two histones are neighbors along DNA within the nucleosome core.

MATERIALS AND METHODS

Isolation and Digestion of Chicken Erythrocyte Chromatin.

Blood was removed from 3-4 week old Babcock B-300 chicks by heart puncture and drained into ice-cold 50 mM EDTA (pH 7.0 at 20°), 0.23 M NaCl, 1 mM PMSF. Methods for isolating nuclei and chromatin by modifications of the procedures of Blobel and Potter¹⁸ and Axel et al.,¹⁹ respectively, have been described previously.^{17,20,21} Chromatin (50 A₂₆₀ units/ml) in 1 mM cacodylic acid, 0.1 mM CaCl₂, adjusted to pH 6.5 with NaOH was digested at 37° to 30% acid-solubility with 5 units of staphylococcal nuclease (11,300 units/mg; Worthington) per A₂₆₀ unit (50 µg) of DNA. Digestion was terminated by addition of 1/10 volume of 0.1 M Tris-HCl (pH 7.5), 15 mM EDTA, 1 mM PMSF.

Preliminary fractionation of digests was accomplished by centrifugation on 34 ml, 5-20% linear sucrose gradients in an International SB-110 rotor at 25,000 rpm for 24 hr (6°). Gradients contained 10 mM Tris-HCl (pH 7.5 at 20°), 0.1 mM Na₂EDTA, 0.1 mM PMSF. Further fractionation was done by centrifugation on 12 ml gradients of the above composition in an SB-283 rotor at 40,000 rpm (6°). Fractions were either dialyzed against 0.01 mM EDTA (pH 6.5), treated with pancreatic RNase A (50 units/ml, Worthington) for 30 min at 37° and lyophilized, or were dialyzed into buffer for physical studies.

Gel Electrophoresis of DNA and Histones. Lyophilized samples for DNA analyses were resuspended at a concentration of 1 µg DNA/µl in SDS gel sample buffer (1% SDS, 1 mM EDTA, 10 mM Tris-HCl, 0.005% bromophenol blue, 10% glycerol, final pH 7.0). Pronase (Calbiochem, B grade) was added (30 µg/ml) and samples were incubated overnight at 37°. Samples were loaded directly onto 15 cm gels of 6% polyacrylamide prepared as described by Loening²² except that the tray buffer and gels were made 0.1% in SDS. Gels were run at 3 ma/gel and scanned at 260 nm. DNA sizes were determined by comparison with Hae III restriction nuclease fragments of PM2 DNA as described previously.²⁰

Samples for histone analyses were resuspended in the above

SDS gel sample buffer made 5% in 2-mercaptoethanol, boiled for 1.5 min, cooled, and loaded onto a discontinuous gel system prepared as described by Laemmli²³ and modified by Bonner and Pollard²⁴ for histones. Gels were electrophoresed at 2 ma/gel, stained with coomassie blue²¹ and scanned at 580 nm.

Circular Dichroism Spectra were obtained on a Jasco ORD/UV-5 instrument with CD attachment and the Sproul Scientific SS20 CD modification.

Thermal Denaturation was monitored using a Beckman Acta CII spectrophotometer. A YSI thermistor probe and thermivolt thermometer were used to give a continuous record of absorbance at 258 nm versus temperature on an X-Y recorder. Temperature was programmed to increase at 1°/min using a Neslab Model TP-2 temperature programmer attached to a circulator bath. Samples were dialyzed exhaustively against 2.5×10^{-4} M EDTA (pH 8.0).

Sedimentation Coefficients were measured using the Beckman Model E analytical ultracentrifuge equipped with UV film optics. Samples in 10 mM Tris-HCl (pH 7.5 at 20°) 0.1 mM EDTA, were centrifuged at 50,740 rpm at 23°. Sedimentation coefficients were determined from boundary midpoint positions and were corrected to $s_{20,w}$ by standard procedures.

RESULTS

Preliminary fractionation on sucrose gradients partially resolved monomeric and oligomeric nucleosomal components plus slow sedimenting acid-soluble oligonucleotides and subnucleosomal particles (Figure 1). No discrete DNA species or histones were found above fraction I. Fraction I contained equimolar amounts of histones H2A and H2B, small amounts of two H1 species (H1A and H1B) plus several discrete DNA fragments 40-70 bp long (not shown). Fraction I was divided into four subfractions, IA to ID, in order of increasing sedimentation, that were further centrifuged on gradients for 26 hr at 40,000 rpm. The gradient profile of fraction ID shown in Figure 2 is characteristic of the separation obtained for all fractions IA to ID. Two peaks are observed, a nonsedimenting component containing only oligonucleotides and a second component with a sedimentation coefficient of 3-4S. Corresponding fractions from the 3S to 4S regions of gradients of samples IA to ID were pooled as shown to

FIGURE 1: Sucrose gradient centrifugation of chromatin digested to 30% acid solubility with staphylococcal nuclease. 160 A₂₆₀ units (8 mg DNA equivalents) were layered onto 34 ml 5-20% linear sucrose gradients containing 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM PMSF and centrifuged at 25,000 rpm for 24 hr at 6° in an International SB-110 rotor.

yield fractions I(AD)1 to I(AD)4.

Electrophoretic analyses of histones and DNA in these fractions (Figure 2) show that I(AD)1 and 2 contained little DNA greater than 40 bp and only traces of equimolar amounts of H2A and H2B. Fractions I(AD)3 and 4 contained discrete DNA fragments 38 and 49 bp long, plus equimolar amounts of H2A and H2B and small amounts of H1A, H1B and two proteins identified as belonging to the HMG class. The fact that the ratio of H2A/H2B remains constant across this portion of the gradient indicates that they are associated as 1:1 dimers. Evidence obtained using a quite different separation technique has shown that the small amounts of H1 and HMG proteins in these fractions are associated with DNA segments separate from the H2 histones (Nelson, Oosterhof and Rill, in preparation).

Peak fraction I(AD)3 was chosen to best represent the (H2A,H2B)-DNA complex and was further characterized and compared

FIGURE 2: Top: Fraction I from the SB-110 run (Fig. 1) was divided, from left to right, into fractions IA through ID that were rerun on gradients in an International SB-283 rotor at 40,000 rpm for 26 hr at 6° (shown is fraction ID only). The fractions A through D were pooled and divided into subfractions I(AD)1 through I(AD)4 for compositional analyses. Fraction 3 was used for physical characterization of the 3.4S particle. Gradient buffer: 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA.

Bottom: Electrophoretic patterns of DNA and histone from fractions I(AD)1 to I(AD)4. The 94 and 156 base pair DNA sizes are from PM2 DNA cleaved with endonuclease R Hae III and coelectrophoresed with the samples. Bands at the tops and bottoms of the gels are UV absorbing impurities. The histone bands designated as H2A and H2B were identified in a separate experiment by including a mixture of calf thymus H3+H4 or H2A+H2B (kindly provided by Dennis Roark) in the samples.

to an 11S nucleosome+nucleosome core fraction obtained after resedimenting the 11S fraction II (Figure 1) at 40,000 rpm for 12 hr. Analytical ultracentrifugation confirmed that the sedimentation coefficients of these two peak fractions were 3.4S and 11.1S, respectively, in agreement with a previous report.¹⁷ The histone/DNA stoichiometry of the 3.4S particles was estimated by electrophoresing equal amounts (A_{260} units) of whole chromatin, the 11S fraction, and the 3.4S fraction on separate histone gels and comparing the total areas under the histone peaks. The total staining areas of all three samples were nearly identical, demonstrating that there is one copy each of histones H2A and H2B per 35-40 bp DNA segment, assuming that there are two copies each of the core histones per 140-160 bp in the 11S fraction or per about 200 bp in whole chromatin.

FIGURE 3: Left: Thermal denaturation profiles of (A) the 3.4S particle treated with Proteinase K (E. Merck, Darmstadt, Germany), (B) the 3.4S particle, and (C) the 11S particle. Samples were in 2.5×10^{-4} M Na_2EDTA (pH 8).

Right: Derivative melting curves for (A) the 3.4S particle treated with Proteinase K, (B) the 3.4S particle and (C) the 11S particle.

Thermal denaturation of DNA in the 3.4S particle occurs predominantly as a sharp transition with a T_m of 61° (Figure 3), considerably higher than the T_m of 44° found for DNA from 3.4S particles treated with Proteinase K, which extensively degrades the histones (see below). Thus these short DNA fragments are bound to, and stabilized by, the H2A, H2B pair, but not to the extent found in the 11S fraction, which has a T_m of 76° (Figure 3).

Other effects of Proteinase K on the 3.4S particle included a decrease in sedimentation coefficient to 2.1S and a decrease in molecular ellipticity at 225 nm (principally due to histones) from -12.5×10^3 to -2.1×10^3 deg-cm²/decimole (Figure 4), demonstrating that the proteins are nearly totally degraded and

FIGURE 4: Circular dichroism spectra of (A) the 3.4S particle, (B) the 3.4S particle treated with trypsin-TPCK (Worthington), and (C) the 3.4S particle treated with Proteinase K. Samples were in 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA. Molecular ellipticities are in terms of moles of DNA phosphate.

FIGURE 5: Circular dichroism spectra of (A) the 11S particle, (B) the 11S particle treated with trypsin-TPCK, and (C) the 11S particle treated with Proteinase K. Samples were in 2.5×10^{-4} M Na_2EDTA (pH 8). Molecular ellipticities are expressed in terms of moles DNA phosphate.

released from DNA by Proteinase K. Most interesting is the observation of a 63% increase in ellipticity at 280 nm (due almost entirely to DNA) upon loss of protein. Qualitatively similar changes in CD spectra (Figure 5) and $S_{20,w}$ were observed upon treatment of the 11S particles with Proteinase K. In contrast, treatment of the 3.4S with particle trypsin caused a significant decrease in sedimentation coefficient (from 3.4S to

2.5S), but virtually no change in circular dichroism either in the region below 240 nm dominated by peptide bond contributions or in the region above 260 nm dominated by DNA (Figure 4), while identical treatment of the 11S particles reduced the circular dichroism at 225 nm slightly and increased the ellipticity at 280 nm to a value intermediate between that of the intact and Proteinase K treated samples (Figure 5).

DISCUSSION

Studies of interactions between specific pairs of histones have demonstrated a strong propensity of histones H2A and H2B to form 1:1 dimeric and oligomeric complexes in solution,²⁶⁻²⁹ thereby leading to suggestions^{30,31} that these two histones are paired in some manner within structural subunits (nucleosomes) of chromatin. That these histones exist in close proximity in chromatin has been amply demonstrated using various cross-linking agents.³²⁻³⁵ Particularly noteworthy is the 80% yield of H2A, H2B dimer reported by Martinson et al.³⁶

The present finding of a chromatin fragment containing approximately 38 bp of DNA associated with equimolar amounts of H2A and H2B confirms directly the close proximity of these histones in chromatin and additionally demonstrates that H2A and H2B are proximate along the DNA. Since this histone pair contains nearly enough basic residues (61, including histidine and α -amino groups) to neutralize the phosphates on a 38 bp length of DNA, and since these particles sediment freely away from larger fragments at low ionic strengths, it appears likely that most, if not all, of the interactions of these histones with DNA are localized and do not extend to distant portions of the nucleosome.

The yield of 3.4S particles from the total digest was not determined directly in these experiments. However, we have previously shown that the two smallest distinct subnucleosomal DNA species from chromatin, now sized as 38 and 49 bp, are together produced in amounts approximately equimolar to DNA of nucleosome core size near the digestion limit (but before precipitation occurs).¹⁷ Examination of gradient profiles in our previous report¹⁷ further shows that the 3.4S and 11S particles are present in about equimolar amounts after extensive digestion.

Other studies have shown that DNA of this size, and particles containing only H2A and H2B, are produced during the digestion of chromatin in intact nuclei by staphylococcal nuclease at an early stage after the appearance of ~200 bp length DNA and nucleosome monomers (ref. 17; also Nelson, Oosterhof and Rill, in preparation). Hence the 3.4S particles are significant products of the digestion and are not artifacts of extensive digestion of chromatin or of the chromatin isolation procedure.

Changes in the circular dichroism spectrum of these 3.4S particles upon treatment with Proteinase K (but not trypsin) show that the H2A, H2B pair alone can alter the optical activity and perhaps the conformation of very short, i.e., local regions of DNA. Thus long range, cooperative histone-histone or histone-DNA interactions may not be required to explain at least some of the unusual optical activity of DNA in chromatin. Furthermore, only the regions of histones not released from DNA by trypsin appear necessary for altering the DNA circular dichroism in the H2A,H2B complexes.

Finally, it is interesting to note that the thermal denaturation and circular dichroism spectrum of both the 3.4S and 11S chromatin fragments (particles) are different from whole chromatin. For example, the 3.4S and 11S particles melt predominantly at 61° and 76°, respectively, while whole chromatin under these conditions melts in at least three and often four phases with T_m 's of approximately 63°, 70° and 81°. ³⁸ Similarly the circular dichroism of DNA in the 11S fraction above 260 nm is reduced compared to that of chromatin while that of the 3.4S fraction in this region is intermediate between that of 11S particles and DNA (even taking into account 20-30% possible contamination by oligonucleotides). Thus perhaps in intact chromatin at the low ionic strengths used in most physical and many other studies, some of the H2A,H2B contacts with other histones (particularly those already weakened by histone modifications) are broken, causing an extension or loosening of this region of nucleosome core DNA with a concomitant increase in susceptibility to nuclease.

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* Author to whom reprint requests should be sent.

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