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Proton NMR Investigation of the Nucleosome Core Particle: Evidence for Regions of Altered Hydrogen Bonding†

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ABSTRACT: Novel ^{1}H nuclear magnetic resonance (NMR) resonances, arising from exchangeable protons and centered at ~11.2 and 10.1 parts per million (ppm), have been observed in the low-field spectrum (10–15 ppm) of the chicken erythrocyte core particle [145 ± 2 base pairs (bp)]. These peaks are located upfield from the normal adenine-thymine (A-T) and guanine-cytosine (G-C) imino peaks characteristic of B-form deoxyribonucleic acid (DNA) and are not observed in free DNA under identical conditions. The appearance of the new peaks is ionic strength dependent and temperature-reversible below 75 °C. At 25 °C, the upfield peak area represents 5% of the DNA base pairs (7 bp), while between 45 and 55 °C, the area increases to 18%, affecting approximately 25 bp. Area increases in the upfield resonances result in a complementary decrease in the A-T and G-C imino peaks found between 12 and 14 ppm. We believe these novel proton signals represent a histone-induced DNA conformational change which involves localized alteration of base pairing in the core particle.

Because the packing ratio within the nucleosomal structure has been determined to be 7, i.e., seven lengths of deoxyribonucleic acid (DNA)[1] contained per length of nucleosome core, conformational states have been proposed for the DNA in the nucleosome which would allow for a tightly coiled structure and would agree with nuclease digestion patterns. Early considerations led to the suggestion that the phosphodiester backbone in the core particle was primarily B form but might contain bends (Crick & Klug, 1975) or kinks (Sobell et al., 1976) occurring at regular intervals. Crick & Klug (1975) proposed a helix which was primarily B form but contained a 98° bend every 20 bp. Sobell (1976) predicted a more drastic alteration of the phosphodiester backbone which resulted in a 40° kink every 10 bp. The 10 bp kink, in this model, was conjectured to be the molecular basis for the 10 bp cutting frequency pattern characteristic of DNase I. Both models predicted base unstacking to occur at the bend. Until recently, no data have supported the presence of any conformationally distinct region (i.e., bend) in the backbone of nucleosomal DNA. Measurements made by ^{31}P NMR (Cotter & Lilley, 1977; Kallenbach et al., 1978; Klevan et al., 1979; Shindo et al., 1980), a technique which is quite sensitive to conformational changes in the DNA helix, suggested only one average nucleosomal conformation, which was not significantly different from the conformation in free DNA. ^{1}H NMR (Feigon & Kearns, 1979) of the imino protons of the A-T and G-C base pairs similarly displayed little difference between the spectra of the core particle and free DNA. Consistent with these results, energy calculations (Levitt, 1978; Sussman & Trifonov, 1978) showed that whereas kinked and abruptly bent DNAs were energetically possible, smooth isotropic bending of DNA changed the local conformation of the phosphodiester backbone only slightly and the resulting increase in energy was small.

Recently, Richmond et al. (1984) have determined the crystal structure of the core particle to 7-Å resolution. Unexpectedly, the nucleosome crystal structure revealed the presence of bent regions located at about 30, 60, 80, and 110 bp which displayed "departure from good base stacking" (Richmond et al., 1984). The lack of detection of such altered regions in solution studies of nucleosomes again raises the question as to whether any such regions of altered structure exist under physiological conditions. The presence of bends

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[1] Abbreviations: NMR, nuclear magnetic resonance; ppm, parts per million; bp, base pair(s) of DNA; A-T, adenine-thymine base pair; G-C, guanine-cytosine base pair; H1, H2, H3, H4, and H5, histone proteins 1, 2, 3, 4, and 5, respectively; DNA, deoxyribonucleic acid; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; CD, circular dichroism; UV, ultraviolet light; U, uridine; D_2O, deuterium oxide; Hz, hertz; MHz, megahertz; Tris, tris(hydroxymethyl)aminomethane; TSP, 3-(trimethylsilyl)propionic acid; Pipes, piperazine-*N,N′*-bis(2-ethanesulfonic acid).

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within the core particle could play both an important functional role in protein recognition and also an important structural role in nucleosomal phasing.

Through the use of ^1H NMR, we report the presence of a conformationally distinct region present in the core particle DNA which is not an artifact of preparation, solution conditions, or instrumentation. The conformation is temperature and ionic strength dependent, and these changes are reversible. The number of base pairs involved in the unusual conformation varies from a minimum of 5–6 (4%) at 25 °C to ~26 (18%) under optimal conditions. In this initial report, we attempt to establish the existence and nature of this new nucleosomal DNA conformation and suggest a likely location for such a conformation in relation to the crystal structure of the core particle.

Materials and Methods

Preparation of Chicken Erythrocyte Core Particles. The preparation of highly homogeneous chicken erythrocyte core particles has been described in detail in Yager et al. (1985). Method II was used, as reported, with no modifications.

Electrophoresis. All procedures for polyacrylamide gel electrophoresis were described in detail in Yager & van Holde (1984).

^1H Nuclear Magnetic Resonance. Fourier-transform spectra were accumulated at 270 MHz from a JEOL GX 270 spectrometer with quadrature detection using 5-mm tubes. Spectral parameters were 20 000 scans of 8192 time domain points, a 10 822-Hz spectral window, and a 0.5-s pulse repetition time for the Redfield 214 pulse sequence (Redfield & Kunz, 1979). A 4-Hz line-broadening function was used to improve the signal to noise ratio. DNA samples were dialyzed into 10 mM Tris, pH 8.0, and 0.1 mM EDTA. An appropriate volume of the DNA solution was next lyophilized and redissolved in 0.5 mL of H_2O and 50 μL of 99.8% D_2O to yield a final DNA concentration of 3.0×10^{-2} M (bp) ($A_{260} = 400$). Each sample contained an internal standard of 0.01% 3-(trimethylsilyl)propionic acid (TSP) which was added solid to each tube. The core particles ($A_{260} \sim 100$) were dialyzed into 10 mM Tris, pH 8.0, and 0.1 mM EDTA and the appropriate NaCl concentration. The dialyzed samples were concentrated further to a final concentration of 3×10^{-2} M (bp) ($A_{260} = 400$) by using an Amicon PM-50 membrane. A buffered D_2O/TSP solution was made by lyophilizing TSP with 10 mM Tris, pH 8.0, and 0.1 mM EDTA and then redissolving in 99.8% D_2O. Concentrated core particles were added directly to the buffered TSP such that the final concentration of internal standard was 0.01% 3-(trimethylsilyl)-propionic acid and the final D_2O content was 10%.

Sedimentation Velocity, Circular Dichroism, and Thermal Melting. Procedures for sedimentation velocity experiments as well as circular dichroism measurements are described in Yager et al. (1985). Thermal melting procedures are described in McGhee & Felsenfeld (1980).

Results

Integrity of the Core Particle. Polyacrylamide gel electrophoresis (Figure 1A) of the native core particle (lane 1) and DNA extracted from the core particles (lane 3) showed an extremely homogeneous preparation which lacked both high molecular weight contamination and any significant subnucleosomal nicking. The DNA isolated from the core particle (lane 3) migrated as a single tight band. Plots of log molecular weight vs. relative mobility (not shown) of the marker DNA fragments were used to determine the molecular weight distribution of the core particle preparation. The relative mobility

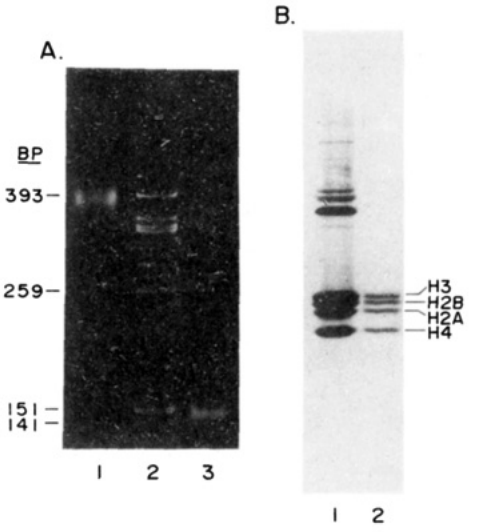

FIGURE 1: (A) Results of gel electrophoretic analysis for the nucleosome core particles (lane 1) and DNA isolated from the core particles (lane 3). Polyacrylamide gels were 1.0 mm thick containing 3.5% polyacrylamide [acrylamide:bis(acrylamide) ratio of 20:1]. Lane 2 contains DNA molecular weight markers from *Cfo*I-cut pBr322. Lane 1 shows 30 ng of the native core particle in 10% glycerol, 10 mM Tris, pH 8.0, and 0.1 mM EDTA. Lane 3 contains 50 ng of the DNA isolated directly from the core particles in standard Laemmli (1970) sample buffer. Gel electrophoresis was performed at 4 °C at 10 V/cm for ~50 min. The gels were stained for 20 min with 1 μg/mL ethidium bromide. (B) Laemmli SDS–15% polyacrylamide gel electrophoresis of the core particle preparation (lane 2) and untreated chicken erythrocyte chromatin (lane 1). Polyacrylamide gels were 0.8 mm thick containing 15% polyacrylamide [acrylamide: bis(acrylamide) ratio of 30:0.8] in the separating gel and 6% acrylamide in the stacking gel. Lane 2 contains 1.0 μg of total protein from our preparation. Lane 1 represented 3.12 μg of chicken erythrocyte whole chromatin. Samples from a core particle solution ($A_{260} = 1.0$) in 10 mM Tris and 0.1 mM EDTA, pH 8.0, were boiled in Laemmli (1970) SDS sample buffer for 1 min, cooled on ice, and loaded onto an Idea Scientific minigel apparatus. Gel electrophoresis was performed at 4 °C at 15 V/cm for 1 h. The gels were stained overnight with 0.25% Coomassie blue and destained in methanol/acetic acid according to the procedure of Laemmli (1970).

of the upper and lower edges of a core particle 10-ng DNA band indicated that the core length ranged from 143 to 147 bp, and is therefore taken as 145 ± 2 bp. Our preparation, shown in Figure 1, displayed no trace of dimer or higher molecular weight oligomer present in any lane. In addition, lanes 1 and 3 display no trace of free dissociated DNA or short oligomers running below the DNA band. The integrity of the intact core particle was confirmed by sedimentation velocity analysis, which determined the sedimentation coefficient of the intact cores in 10 mM Tris, pH 8.0, and 0.1 mM EDTA to be 10.8 ± 0.2 S at 20 °C, in excellent agreement with previous studies (Tatchell & van Holde, 1979; Ausio et al., 1984). Analysis of protein on standard Laemmli gels (Figure 1B) demonstrated that our preparation was extremely pure, lacking both H1 and H5 and any significant amounts of non-histone proteins. The circular dichroism spectrum, in the region from 300 to 260 nm, displayed the characteristic features of native nucleosomes: a small negative peak at ~295 nm, a double peak exhibiting a maximum ellipticity of 1400 ± 200 deg·cm^2·mol^{-1} at 282.4 nm, and a zero crossover point at ~270 nm. Midpoints of the thermal melting profiles (Figure 4A) in 10 mM Tris and 0.1 mM EDTA, pH 8.0, were in excellent agreement with previously reported results (Weischet et al., 1978; McGhee & Felsenfeld, 1980).

^1H NMR. The ^1H NMR imino proton spectral region at 25 °C for both the chicken erythrocyte core particle and chicken erythrocyte DNA isolated directly from the core

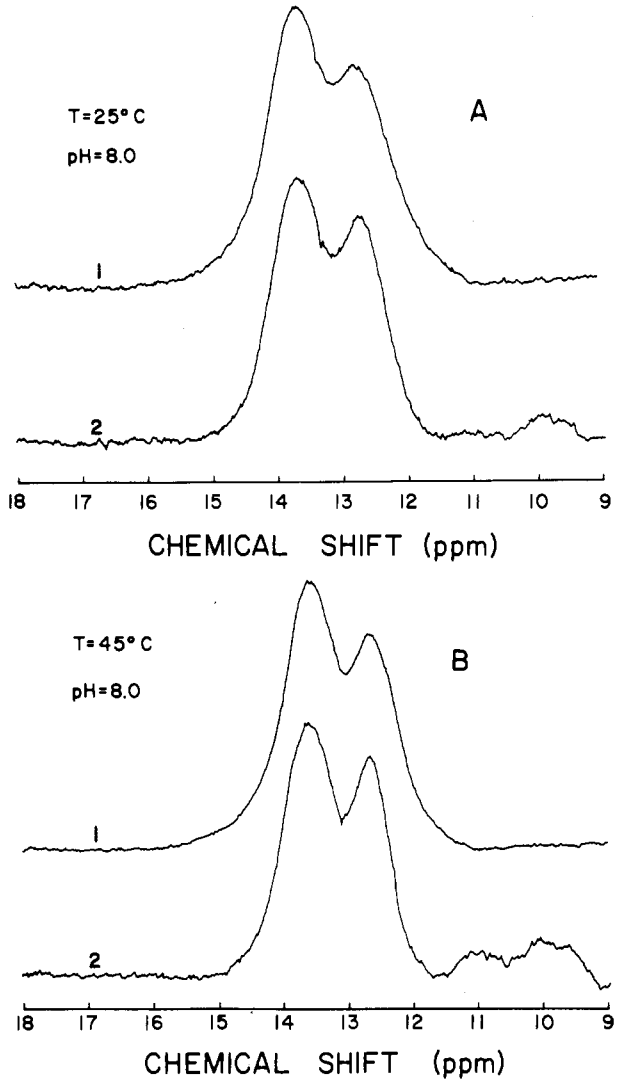

FIGURE 2: (A) ^{1}H NMR spectra of the exchangeable protons for free DNA (1) and chicken erythrocyte core particles (2) in 10 mM Tris, pH 8.0, and 0.1 mM EDTA. Fast Fourier transformed spectra were accumulated at 25 °C at 270 MHz with quadrature detection using 5-mm tubes. Spectral parameters were 20 000 scans of 8192 time domain points, a 10 822-Hz spectra window, and a 0.5-s pulse repetition time (0.1-s pulse delay) for the Redfield 214 pulse sequence. The concentration of the core particles was 6×10^{-2} M (bp) ($A_{260} = 400$) in a volume of 0.5 mL. Spectra were recorded relative to an internal standard of 0.01% 3-(trimethylsilyl)propionic acid (TSP). (B) Same as (A) but the temperature was increased to 45 °C.

particles is shown in Figure 2. Examination of Figure 2A reveals the partially resolved imino resonances which are typical for *native* DNAs. The downfield A-T peak at ~13.6 ppm and the upfield G-C peak at ~12.6 ppm for free DNA differ little from the A-T (13.7 ppm) and G-C (12.8 ppm) peaks observed for the core particle. These results are in good agreement with Feigon & Kearns (1979), who measured similar ^{1}H NMR spectra for a variety of native DNAs and calf thymus core particles. We point out here that due to the solvent suppression pulse sequence used, distortion of the peripheral chemical shift range can produce a "rolling" base line which makes peak phasing more difficult than a normal ^{1}H NMR experiment. Under our conditions, a fairly constant excitation pulse intensity is obtained in the 10–16 ppm range (Redfield et al., 1975). In reporting our spectra, we attempted to phase the peaks in a manner which yielded symmetrical peak shape, flat base line and produced peak areas representative of the known A-T and G-C percent composition. However, because of the broad ^{1}H NMR peaks and the solvent

Table I: Calculated Areas of Imino Proton Peaks for Calf Thymus and Chicken Erythrocyte DNA or Nucleosomes

samples[a]	T (°C)	% large[b]	% small[c]	% A-T[d]	% G-C
calf thymus DNA	25	100		55	45
chicken erythrocyte DNA	25	100		56	44
chicken erythrocyte core particles	25	100[e]	0	55	45
	25	95[f]	5	51	44
	15	97	3	53	44
	25	95	5	51	44
	65	89	11	51	38
	45	86	14	50	35
	55	82	18	47	35
	65	79	20	[g]	
	70	76	24		
	75	63	37		

[a] The buffer used for all NMR experiments was 10 mM Tris, pH 8.0, and 0.1 mM EDTA. The concentration for all samples was 2×10^{-2} M ($A_{260} \sim 400$). [b] Large peaks = sum of the known imino A-T and G-C resonances between 12 and 14 ppm. [c] Small peaks = sum of the small upfield resonances between 10 and 11.5 ppm. [d] The percent A-T was determined by the ratio of the A-T peak area to the total (sum of large and small peak areas) peak area. The A-T peak area was determined by drawing the isolated A-T resonance assuming a standard Lorentzian distribution. The peak maximum was taken as the peak midpoint, and the base line was drawn through the center of the base-line noise. Points on the left side of the A-T curve were measured, and the A-T resonance was completed by drawing equidistant points to the right of the peak midpoint. The A-T peak area was determined by cutting and weighing. [e] Total area = sum of large peaks. [f] Total area = sum of large + small peaks. [g] Area loss began at 65 °C. No percent composition for the large imino peaks is reported after 55 °C.

suppression technique used, the individual (A-T, G-C) peak areas can be determined no better than ±10%. No nonlinear base-line manipulations were used in plotting the spectra so that area measurements could be more accurate. The method was standardized by taking the spectra of sonicated calf thymus DNA and chicken erythrocyte DNA which have known base compositions of 57% A-T and 43% G-C. The results in Table I revealed that for both the calf thymus and chicken erythrocyte DNAs, the imino areas are in excellent agreement with base composition results determined by thermal melting (Marmur & Doty, 1962).

For the core particle, we observed (Figure 2A) the presence of a small intensity in the upfield region of the spectrum at around 10.0 ppm; this was not seen for the corresponding DNA. The relative base composition was calculated for the core particle (Table I) with and without the inclusion of these small peaks. The area of the small upfield resonance was approximately 5% at 25 °C, which was equal to the difference between the known A-T content of chicken erythrocyte DNA and the value determined by calculation of the A-T imino peak area (when the small peaks were included in the total area). Upon increasing the temperature for the core particle, the small peaks, seen in Figure 2A, increased in area and are clearly seen as an upfield set of peaks centered at 11.2 and 10.1 ppm (Figures 2B and 3). It is important to note that no intensity is observed in this spectral region for free DNA at all temperatures under the same conditions. The DNA is identical in length and base composition with the core particles used in this experiment. Between 15 and 55 °C, the area in the upfield signals significantly increased. The small peaks, which represented only 3% at 15 °C, reached a maximum between 37 and 55 °C involving ~18% of the total peak area. In this temperature range (15–60 °C), the integrated intensity (total area) of the ^{1}H NMR spectra between 9 and 14 ppm remained constant. Therefore, since no loss of area due to bulk solvent exchange occurred, we calculated the relative A-T and

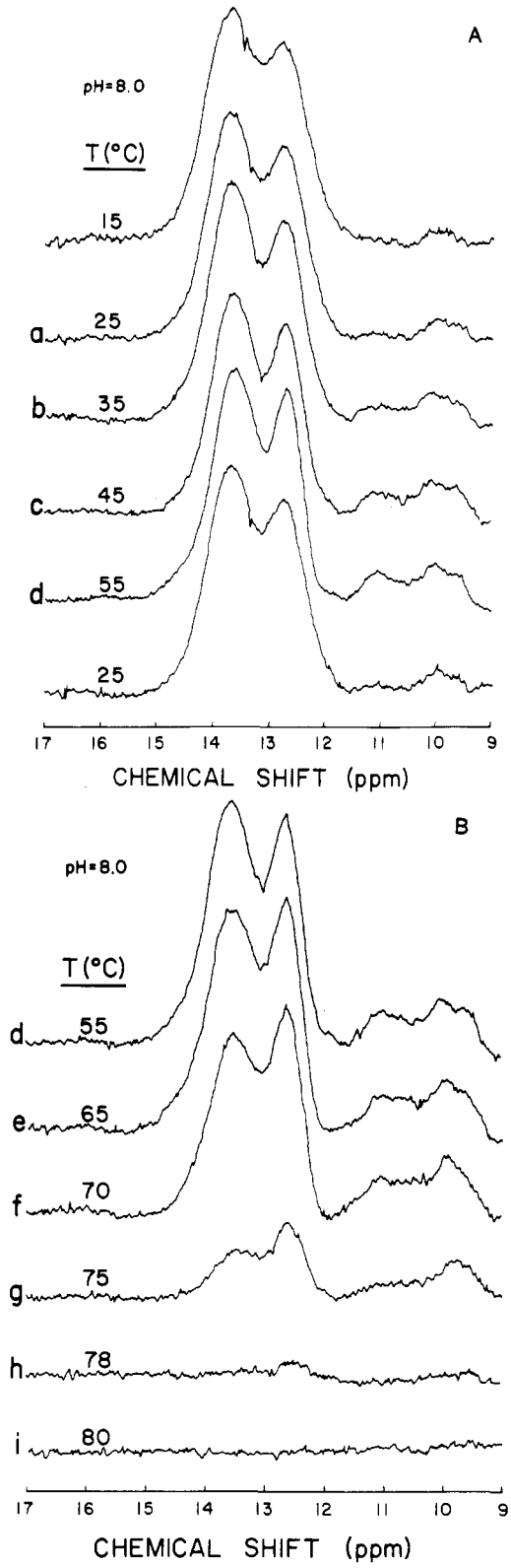

FIGURE 3: (A) ^{1}H NMR spectra of the chicken erythrocyte core particle in 10 mM Tris, pH 8.0, and 0.1 mM EDTA at 15, 25, 35, 45, and 55 °C. The 25 °C spectrum at the bottom of (A) is the same sample which, after being heated to 55 °C, was cooled to 25 °C. Small letters refer to the UV spectrum position in Figure 4A. All spectral parameters and conditions are listed in Figure 2A. (B) Same as (A) but at 55, 65, 70, 75, 78, and 80 °C.

G-C percentages for the large imino peaks. Because the total area remained constant, the small and large peaks appeared related by the fact that an increase in the small peak area was concomitant with a decrease in the large imino peak area. Thus, an increase in temperature induced a conformational change which was detected in the upfield region of the ^{1}H NMR spectrum at the expense of the normal B-form peaks observed between 12 and 14 ppm. From 60 to 70 °C (Figure 3B), we began to see loss of area due to solvent exchange which was especially apparent in the A-T peak of the large imino resonances. In this same region, the area of the small peaks remained largely unaffected. At 75 °C, a substantial portion of the large peaks is in fast exchange with solvent. Although some apparent loss of area has also occurred for the upfield resonances, the majority of these proton signals remain in slow exchange. Above 78 °C, we observed total loss of area for all peaks. The chemical shifts of both the large and smaller peak maxima were shifted only slightly ($\leq$0.08 ppm) upfield when the temperature was increased. An important feature of this temperature effect was that the appearance of the new peaks was completely reversible. When the core particles were gently heated to 55 °C and cooled, the peak areas decreased again to 4% of the area at 25 °C (Figure 3A), identical with the value before heating. During both the heating and cooling processes, the peak areas and shapes were identical at the respective temperatures.

The observation of these upfield peaks was unexpected, since no Watson–Crick-type imino hydrogen-bonded protons for any natural or synthetic DNA have been reported in this chemical shift region. To ensure that the small peaks were not an artifact of preparation or solution conditions used, we examined the exchangeable ^{1}H NMR spectrum for the core particle from several different nucleosome preparations as a function of buffer (Tris, Pipes), and with or without the presence of TSP, the internal reference which was included in each sample. The ^{1}H NMR spectrum for the Tris or Pipes buffer showed no intensity from 9 to 19 ppm, and neither the presence nor the area of the upfield peaks was affected by choice of buffer or elimination of the TSP standard. To ensure that the new signals did not represent protein hydrogens, we examined the ^{1}H NMR spectra of DNA/tetralysine complexes at various temperatures. The association of the tetrapeptide with DNA, an interaction which bears some similarity to histone/DNA electrostatic interactions, showed *no* intensity between 9 and 11.5 ppm at any temperature point measured. (We have not been successful, at present, in obtaining ^{1}H NMR spectra for DNA complexes with oligoarginine or larger oligolysine peptides due to precipitation problems which occurred at the low ionic strength and high concentrations used in the NMR experiments.) Additionally, we monitored the fractional exchange of the larger known imino peaks and the small peaks with D_2O. A core particle sample ($A_{260} \sim 400$) from the same preparation as is shown in Figure 1 was heated to 35 °C. The area of the small peaks was identical with the value reported in Table I. Quickly, Tris-buffered D_2O was added to the core particle sample so that the final concentration of core particles was $A_{260} \sim 200$ (this concentration will yield a good spectrum) and the final percent of D_2O was 50. The sample was remeasured within 15 min. We observed a 50% reduction in area for both the large and small peaks.

We confirmed that the upfield resonances were a direct result of the histone/DNA interactions by an independent experiment. Increase of NaCl concentration to 2 M, within the same sample of core particle, resulted in the disappearance of the peaks from 9 to 11.5 ppm. Under physiological conditions, 0.2 M NaCl and 37 °C, the small peaks involved 4% of the total imino area, i.e., 5–6 DNA bp.

UV and Circular Dichroism Thermal Melting Studies. The results of Shindo et al. (1980) and Simpson & Shindo (1979)

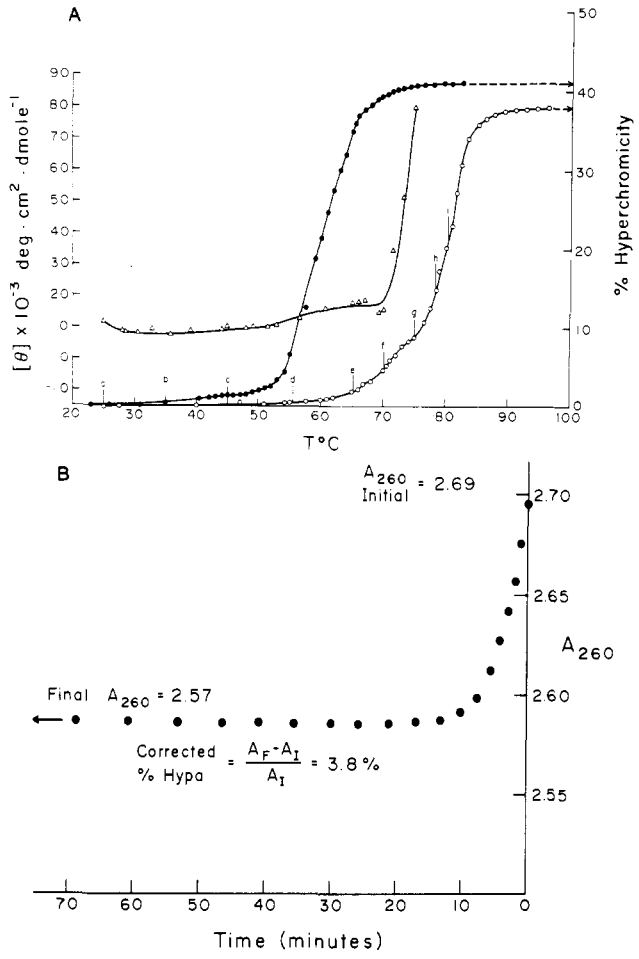

FIGURE 4: (A) Thermal melting profiles for chicken erythrocyte core particles and the free DNA isolated directly from the core particles. The melting profiles were monitored by both the UV absorbance change at 260 nm and the molar ellipticity (CD) changes at 275 nm: (●) UV melting profile for chicken erythrocyte DNA; (○) UV profile for chicken erythrocyte core particles; (△) circular dichroism profile for the core particles. The buffer used for all experiments was 10 mM Tris, pH 8.0, and 0.1 mM EDTA, and the concentration used for UV and CD melting was, respectively, 2.2×10^{-5} M (bp) (A_{260} = 0.3) and 5.3×10^{-5} M (bp) (A_{260} = 0.7). All measurements were made in rectangular or cylindrical 1-cm quartz cells. (B) Results for the absorbance decrease vs. time for ionic strength dissociation of the nucleosome core particle at 37 °C in 10 mM Tris, pH 8.0, and 0.1 mM EDTA. The absorbance decrease is plotted after addition of Na^+ to 2 M.

demonstrated that the high concentrations used in the NMR experiment did not hinder the ability to detect solution conformation. Melting profiles at high concentration (NMR) were identical in interpretation with those determined by UV and CD, techniques which measure changes at much lower concentration. Therefore, to further elucidate the conformational state of the core particle in the temperature region where the new imino peaks were present, we measured the UV and circular dichroism melting profiles in 10 mM Tris, pH 8.0, and 0.1 mM EDTA. Both melting profiles were in excellent agreement with previous studies. The ^{1}H NMR spectra at various temperatures can easily be assessed for base unstacking (Figure 4A). The temperature corresponding to each ^{1}H NMR spectrum is marked a–j in the UV melting profile. In the range from 20 to 55 °C, we observed no hyperchromicity change at 260 nm for the core particle. Similarly, little change (a slight decrease) in the molar ellipticity at 275 nm was seen in this temperature range. Initially, it seemed there was no evidence for any significant alterations in base stacking which might account for the presence of the unusual proton resonances. It occurred to us, however, that we were only able to measure changes from the initial or native state of the core particle. Although no conformational changes detectable by UV or CD occurred in the core particle from 20 to 55 °C, we could say nothing about the initial state. Information about base stacking differences in the core particle could be determined, however, from the comparison of the hyperchromicity values for free DNA relative to the nucleosomal DNA. Examination of Figure 4A reveals that, indeed, there is a 3% difference in the total hyperchromicity values for the two forms of the DNA. This hyperchromicity difference has been previously observed (McGhee & Felsenfeld, 1980; Tatchell, 1977) but has remained unexplained. Independent melting of histone octamers resulted in an absorbance change of ~0.03%, and, therefore, we determined that the histone octamer did not significantly contribute to the melting profile. If a hyperchromicity difference really existed, then dissociation of the protein from the core particle should produce a hypochromic effect. The following experiment was performed: we measured the absorbance at 260 nm of a sample of chicken erythrocyte core particles and the DNA isolated directly from the same preparation. Both samples had a volume of 3 mL, were dialyzed into an identical buffer, and were equilibrated to 37 °C. After measurement, we added solid NaCl to each sample such that the final concentration of Na^+ was 2 M. Figure 4B follows the decrease in absorbance at 260 nm which accompanied the dissociation. The results showed that a ~4% hypochromic effect was observed, in good agreement with thermal melting results. Finally, to show that the observed hypochromism was not the effect of ionic strength increases, we measured the absorbance change which accompanied the removal of the protein by Pronase digestion under low ionic strength conditions. Again, Pronase removal of the histones resulted in a hypochromic effect of ~5%.

Discussion

We have observed by ^{1}H NMR the existence of an unusual set of low-field proton resonances for the chicken erythrocyte core particle under physiological conditions. We have confirmed that these peaks are not an artifact of preparation or any solution condition used in the NMR experiment. The resonances do not represent simple end-fraying effects or strand nicking, since upfield peaks are not seen for the corresponding free DNA isolated from these particles. The novel ^{1}H NMR peaks are located upfield from the A-T and G-C imino hydrogen-bonded proton signals observed for native core particles or B-form DNA and are *not* observed in free DNA spectra under identical buffer and temperature conditions. Further, the new peaks are only seen in H_2O solutions by using solvent suppression techniques, and the appearance of these peaks results in no loss of the total signal area from 15 to 55 °C. We conclude from these results that the upfield peaks represent hydrogen-bonded proton resonances which are unique to the DNA/histone complex. By their shape and position, it is immediately tempting to identify these upfield peaks as imino protons which have an altered environment due to histone association. This interpretation is supported by the fact that these peaks increase at the expense of the normal imino proton peaks. However, several other interpretations must be considered. In addition to imino protons, the peaks could be (i) hydrogen-bonded protons from histones, (ii) amino hydrogen-bonded protons from DNA bases, or (iii) a combination of the above. Fortunately, examination of the data eliminates at least one of these possibilities. First, let us consider the possibility that the new proton resonances arise from protein hydrogens. Proton spectra for the histone core, in 2 M NaCl

and in the absence of DNA (Cary et al., 1978; Nicola et al., 1978; Walker, 1984), show no peak intensity in the proton spectrum from 10 to 15 ppm. Thus, the spectrum of the intact histone core itself does not give rise to any low-field spectrum. In 2 M NaCl, the histone core remains folded as an octamer similar to its native state (Beaudette et al., 1981; Godfrey et al., 1980) and, in the absence of DNA, some of the surface-exchangeable protons could be in rapid exchange with H_2O and would not be visible in the low-field ^1H NMR spectrum. The absence of intensity between 10 and 15 ppm for the histone core is the result we might predict for the free protein. If we now associate the histone core with the DNA, some protons, initially in fast exchange with H_2O, could be trapped by hydrogen bonding. Since the exchange rate would be greatly reduced, they could now be seen in the ^1H NMR spectrum. If we assume that the interactions which stabilize the native core particle structure are primarily arginyl/lysyl-type interactions, and if the upfield peaks represent such protons, their number should *decrease* as the temperature is raised (Walker, 1984; McGhee & Felsenfeld, 1980; Weischet et al., 1978). Assuming that a maximum number of contact points is present in the native state of the core particle, it is difficult to explain the *increase* in peak intensity observed from 15 to 55 °C. We would have to conclude that 6–25 new protein contacts were forming upon temperature increase. DNA/tetralysine complexes give no intensity between 9 and 11 ppm in this same temperature range. Additionally, the rapid exchange with D_2O of the small peaks was indistinguishable from that of the imino protons of the core particle DNA. Mandal et al. (1979) have shown that, under our conditions, imino proton exchange with bulk solvent occurs on a millisecond time scale (~200 ms). Thus, the rapid exchange observed by ^1H NMR supports the idea that the upfield area we observe is due to exchangeable DNA protons and not to protein hydrogens. For free histones, the only proton resonance found near 9–11 ppm is the amide proton in the histone backbone. This resonance is located at ~8.2 ppm (Nicola et al., 1978). Free from contact with DNA, this resonance is clearly upfield to the 9–11 ppm range. If the amide proton were actually hydrogen bonded directly to the phosphate backbone or a base, then deshielding of the proton could result in a slight downfield shift. However, if the upfield ^1H NMR area represented an amide protein hydrogen complexed to DNA, then we would expect that the exchange rate would be much longer than the few minutes observed by NMR (McCarthy et al., 1984; Englander et al., 1972). We conclude that the protons involved are exchangeable DNA protons and, in double-stranded DNA, the only candidates are the G,T imino hydrogen-bonded protons or the A,G,C amino hydrogen-bonded protons. Let us now consider the case of the amino proton. Exchangeable amino protons have been observed in several reports (Fazakerley et al., 1984; Wong et al., 1972; Clore et al., 1985). While predictions have been made that resonances in the range from 9 to 11 ppm could represent amino protons associated with unusually strong hydrogen bonding (Kearns, 1977; Patel, 1976), no amino resonances have been observed in this region in DNA under our conditions. Conclusive identification of exchangeable amino protons has been detected only in the region from 6.5 to 8.5 ppm for synthetic oligonucleotides (Clore et al., 1985) and $d(GC)_3$ (Fazakerley et al., 1984) under normal conditions. The lack of any conclusive assignment for amino resonances from 9 to 11 ppm leads us to favor the remaining interpretation. We propose that the small peaks seen at 10.1 and 11.2 ppm represent imino protons which have altered or disrupted hydrogen bonding. If the hydrogen bonding is actually disrupted, then protein association must protect the protons from rapid solvent exchange. Such an interpretation is clearly the most likely, not only in view of the shape, position, and exchange rate of the peaks but also because many such altered imino peaks have already been identified in tRNAs [see Reid & Hurd (1979) and references cited therein] and synthetic oligomers [see Kearns (1984) and references cited therein]. In all reported cases, imino peaks found between 9 and 11 ppm represented only two hydrogen classes: (i) protons with nonstandard base pairing, or (ii) protons which lack base pairing but remain in slow exchange. Since the imino protons in double-helical DNA are already found upfield (relative to their intrinsic position) by ring current shifting and since base pair ring current shifts at 3.4 Å are generally less than 1 ppm, the observed upfield positions of imino protons are not likely to result from shielding effects due to adjacent base pairs. In fact, we would expect that departure from good stacking would result in the opposite effect, i.e., deshielding and a downfield shift. Thus, the upfield position of the peaks does indicate that actual alteration of hydrogen bonding occurs in the nucleosome, which might involve base unstacking. Although we have not yet assigned the individual upfield resonances, we interpret the presence of two to three upfield peaks to be altered G or T imino protons which differ in their structural environment (position or geometry).

We now consider whether disruption or alteration of base pairing within the core particle necessarily alters the geometry of the backbone. In the case of the 12 bp complementary fragment with a T-T wobble pair insert, Haasnoot et al. (1979, 1980) argued that since the chemical shift of the inserted T-T imino proton was 1 ppm to higher field (more shielded) than that observed for the imino proton in single-stranded dTTTT, the T-T imino proton was required to be ring current shifted and within the shielding cone of the neighboring base. Thus, the T-T mismatched pair tended to be incorporated within the stack, despite its lack of Watson–Crick hydrogen bonding. Similar conclusions have been reached for G-T wobble pairs (Early et al., 1978; Patel et al., 1982). In addition, theoretical calculations [see Rein (1983) and references cited therein] have led to the prediction that the existence of wobble pairs within B-form structures is energetically feasible. Thus, our data appear to support the idea that the nucleosome core particle, while maintaining an overall B-form geometry, has regions of altered hydrogen bonding. These results are supported by the absorbance change differences (Figure 4) which indicate that a minimum of 5 bp of DNA may already exist in an altered manner within the native core particle structure relative to free DNA.

The absorbance differences observed for the core particle are the sum of the effects of any regional alterations plus overall conformational alterations. The overall conformational alterations in free DNA on winding around the histone octamer have been monitored by CD (Weischet et al., 1978). This overall conformational change can be mimicked by DNA in high ionic strength, and the change in winding has been shown to be decreased by 0.2 bp/turn (Baase & Johnson, 1979). Furthermore, Edmondson & Johnson (1985) have shown that the overall conformational change mimicked by DNA in high ionic strength corresponds to an absorbance *decrease* of 6%. We have measured the total absorbance difference (regional + overall conformational changes) for the core particle relative to free DNA to be an *increase* of ~4%. This implies that the regional or structural alterations alone correspond to an absorbance increase of ~10%. This rather

large difference is not apparent in the measurable extinction coefficient of the core particle because it is masked by the overall winding conformational change. It is not clear, at present, whether the absorbance difference represents full base unstacking of base pairs or a less extreme conformational change. The data seem to support the latter interpretation. The increase in our upfield imino area does not result in any observable hyperchromicity in the UV spectrum from 25 to 55 °C.

Where are the regions of altered hydrogen bonding located? The ^{1}H NMR melting profile shown in Figure 3 demonstrates that most of the intensity of the upfield peak is maintained up to 75 °C, a temperature at which much of the major imino area has been lost. Under our conditions, the first melting phase occurs between 65 and 74 °C. The mechanism of nucleosome melting, proposed by Weischet et al. (1978) and experimentally verified by Simpson (1979), predicts that, in the first phase of melting, the 20 bp at each end of the core particle dissociate and denature before the interior 100 bp begin to melt. If the upfield peaks represented altered hydrogen bonds located at the end of the core particles, then these peaks should disappear in the first melting phase due to solvent exchange at the melted ends. Our results, therefore, suggest that the altered hydrogen bonding probably occurs toward the interior 100 bp of the core particle, i.e., between 20 and 125 bp from the ends.

Two explanations seem to fit our data. First, if the core particle melting represents sequential melting from the ends of the core particle, then the upfield peaks could represent imino protons altered due to an intermediate stable complex (a complex fraying effect) of histone protein with single-stranded DNA ends at the edge of the double-helical region. This intermediate complex would have to form maximum contact prior to the first melting phase of the core particle (55–75 °C) since maximum upfield area is observed at ~55 °C. Further, as the core particle DNA melted, the complex would have to move along in a zipper effect at the interphase between double- and single-stranded DNA and remain unchanged in environment and number from 55 to 70 °C where the upfield area is approximately constant. We feel that this model is complicated and unlikely on the basis of other experiments. Under our conditions, from 55 to 70 °C at least 20 bp have melted from both ends of the core particle and are not further complexed with protein. It seems that significant mobility and rearrangement of histones would be required within the first melting phase in order to maintain maximum contact from 55 to 70 °C. Weischet et al. (1978) measured the CD change at 230 nm due to protein conformational change during thermal denaturation and found that *no* change was observed for the core histones within the first phase. Only in the second phase did extensive change occur. We favor the following interpretation.

From X-ray diffraction, Richmond et al. (1984) have identified the presence of sharply bent regions within the core particle structure at ~30, 60, 80, and 110 bp. These regions are reported to display departure from "good" stacking which is spread over several base pairs at each bend. Although the authors were not able to report more detail on the geometry or the degree of stacking deviation, the existence of these regions clearly could explain the small upfield imino proton peaks. Partial unstacking of the base pairs and DNA bending could easily result in some shielding and alteration of the imino proton bonding. Under the conditions of crystal formation, between 6 and 12% (assuming 2–3 bp affected) of the DNA base pairs are involved in bending. Our results indicate that, depending on the ionic strength and temperature, between 5% and 20% of the imino protons are involved in the unusual conformation. Loss of upfield peak area due to melting initially occurs at ~75 °C, just at the transition from the first to the second melting phase of the core particle. This correlates well with the 30 and 110 bp bent regions. The remaining area is not lost until all the DNA is melted (>78 °C). Again, this appears to correlate with the 60 and 80 bp bent regions at the interior of the core particle near the dyad axis. Further, since the bends are located near regions of extensive histone contact, the thermal stability of the altered regions can be explained. Tight binding of histones prohibits the normal thermal fluctuations which result in base pair opening motions that allow imino proton exchange with bulk solvent. These correlations suggest that the ^{1}H NMR upfield peaks may arise from regions of DNA bending within the core particle structure, in solution. To elucidate further the nature of the upfield ^{1}H NMR resonances, we are currently conducting further investigation on synthetic core particles reconstituted with poly[d(A-T)]·poly[d(A-T)] and core particles devoid of histone tails.

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Preparation of Catalytically Active Cytochromes P-450 by Antigen Exchange on Monoclonal Antibody Based Immunoadsorbents†

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ABSTRACT: Catalytically active cytochromes P-450 have been prepared by monoclonal antibody (MAb) directed immunopurification using an antigen-exchange technique. Immunoaffinity-purified cytochromes P-450 that require denaturants for efficient desorption from the immunoaffinity matrix, although significantly lacking in catalytic activity, were found to retain epitopic structural integrity as probed by radioimmunoassay using MAbs to 3-methylcholanthrene and phenobarbital-induced rat liver cytochromes P-450. These denatured cytochromes P-450 were capable of displacing from the immunoaffinity matrices epitopically related cytochromes P-450 that retained aryl hydrocarbon hydroxylase and 7-ethoxycoumarin *O*-deethylase activities. Such epitope-specific exchange of denatured for native antigen on a solid-phase matrix containing a MAb may be generally applicable to preparation of proteins with the retention of activity.

The cytochromes P-450 metabolize a variety of xenobiotic and endogenous compounds, including drugs, carcinogens, and steroids (Coon et al., 1980; Gelboin, 1980; Lu & West, 1980; Sato & Kato, 1982). The multiplicity of cytochrome P-450, which has been demonstrated by a variety of biochemical and immunological methods (Lu & West, 1980), is responsible for its broad spectrum of substrate specificity and reactivity. The metabolic fate of cytochrome P-450 substrates therefore depends on the type and amount of the cytochromes P-450 that are present. Progress in distinguishing closely related isozymes has been limited, however, by the difficulties encountered in purification of the individual isozymes and their overlapping enzymatic specificities.

As an approach to the multiplicity problem, we have prepared and characterized several panels of monoclonal antibodies (MAbs)[1] to different cytochromes P-450 (Park et al., 1980, 1982a,b, 1984). MAbs target specific epitopes on the antigen (Yelton & Scharff, 1981) and when coupled with

† This work is dedicated to the memory of Prof. Sherman Beychok.

[1] Abbreviations: MAb, monoclonal antibody; RIA, radioimmunoassay; MC, 3-methylcholanthrene; PB, phenobarbital; MC-P-450, major form of MC-induced rat liver cytochrome P-450; PB-P-450, major form of PB-induced rat liver cytochrome P-450; glycine-eluted P-450, cytochrome P-450 polypeptide eluted from immunoadsorbent with 0.1 M glycine (pH 3.0); PBS, phosphate-buffered saline (5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl, pH 7.4); AHH, aryl hydrocarbon hydroxylase; ECD, 7-ethoxycoumarin *O*-deethylase; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kDa, kilodalton; BCA, bicinchoninic acid.