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Articles

Binding of Ethidium to the Nucleosome Core Particle. 1. Binding and Dissociation Reactions†

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ABSTRACT: We have examined binding properties of and dissociation induced by the intercalating dye ethidium bromide when it interacts with the nucleosome core particle under low ionic strength conditions. Ethidium binding to the core particle results in a reversible dissociation which requires the critical binding of 14 ethidium molecules. Under low ionic strength conditions, dissociation is about 90% completed in 5 h. The observed ethidium binding isotherm was corrected for the presence of free DNA due to particle dissociation. The corrected curve reveals that the binding of ethidium to the core particle itself is a highly cooperative process characterized by a low intrinsic binding constant of $K_A = 2.4 \times 10^4$ M^{-1} and a cooperativity parameter of $\omega = \sim 140$. The number of base pairs excluded to another dye molecule by each bound dye molecule (n) is 4.5. Through the use of a chemical probe, methidiumpropyl-EDTA (MPE), we have localized the initial binding sites of ethidium in the core particle to consist of an average of 27 ± 4 bp of DNA that are distributed near both ends of the DNA termini. MPE footprint analysis has also revealed that, prior to dissociation, the fractional population of core particles which bind the dye (f) may be as low as 50%. Comparison of the binding and dissociation data showed that the cooperative maximum of the binding curve occurred at or near the critical value, i.e., at the point where dissociation began. The data were used to generate a detailed model for the association of ethidium with chromatin at the level of the nucleosome.

Ethidium is a polycyclic aromatic dye which binds by intercalation between the base pairs (bp)[1] of DNA (Lerman, 1961, 1963, 1964; Fuller & Waring, 1964; LePecq & Paoletti, 1967; Waring, 1970). The binding properties of intercalating dyes are of particular interest since compounds of this class have been associated with a wide range of biologically important processes such as mutagenesis (McCann et al., 1975), carcinogenesis (McMahon, 1986; Waring, 1981; Hajduk et al., 1978), and chemotherapy (Wilson & Jones, 1981; Waring, 1972). Ethidium binding to free DNA displays little base pair specificity (Muller & Crothers, 1975) but is preferred at purine–pyrimidine (5′–3′) sequence sites (Reinhardt & Krugh, 1978; Kastrup et al., 1978). X-ray studies of ethidium complexed with complementary dinucleotides reveal that the phenyl and ethyl substituent groups protrude into the minor groove while the amino groups are adjacent to the oxygens of the sugar–phosphate chains on opposite strands (Tsai et al., 1977; Jain et al., 1977). Ethidium unwinds the DNA base pairs at the intercalation site by 26° (Wang, 1974; Pulleyblank & Morgan, 1975; Baase & Johnson; 1979; Jones et al., 1980), inducing a slight bending of the double helix toward the major groove (Tsai et al., 1977), and displays moderate binding affinity to DNA ($K = 10^4$–10^6 M^{-1}), depending on the ionic strength (Quadrigfolio et al., 1974).

While extensive investigation has been undertaken to elucidate the base specificity, sequence requirements, and affinity

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[1] Abbreviations: bp, base pair(s); EDTA, (ethylenedinitrilo)tetraacetic acid; MPE, methidiumpropyl-EDTA-Fe(II); DNA, deoxyribonucleic acid; DTT dithiotreitol; Tris, tris(hydroxymethyl)aminomethane; H2A, histone protein 2A; H2B, histone protein 2B; pBr322, plasmid pBr322; Hpa II (type 2), restriction endonuclease; SDS, sodium dodecyl sulfate.

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which characterize the binding of many intercalating compounds to free DNA, little is known about how chromatin structure modifies their binding or how dye binding affects chromatin structure. In vivo, eukaryotic DNA is condensed into various levels of packaged structures, the most "simple" being a length of DNA associated with eight histone proteins, the nucleosome core particle. Yet even at this most basic level of packaging, little agreement has been reached concerning the mechanism of dye binding. Wu et al. (1980) reported that the binding of ethidium bromide to the chicken erythrocyte core particle displayed negative cooperativity, typical of neighbor exclusion binding seen for free DNA. Apparent binding affinity varied with the ratio of moles of bound dye per mole of DNA base pair (ν); binding of the dye was strongest at very low ratios, and binding affinity gradually decreased in strength. These authors reported that at low ratios of dye to nucleic acid bp, the binding affinity of ethidium to the core particle ($\sim 10^7$ M^{-1}) was an order of magnitude higher than for DNA ($\sim 10^6$ M^{-1}) under similar ionic strength conditions. They also reported that the binding of the dye proceeded without loss of the histone proteins as high as ν = 0.2.

In the model of Wu et al., the association of the histones to DNA resulted in *increased* affinity of the dye for its nucleic acid substrate relative to free DNA. The initial high affinity of the dye to the core particles was attributed to the coupling of dye binding to the release of helical stress induced by the supercoiling of the DNA helix about the histone octamer core. Implicit in the argument was the assumption that the tight binding of histones produced the topological constraints required for a linking number relationship to hold. In contrast, Erard et al. (1979) reported that the binding of ethidium bromide to the core particle was a positively cooperative process. Binding of the first molecule favored binding of the next; consequently, the affinity of the dye was lowest at very low ratios, but gradually increased up to ν = 0.10. Above ν = 0.10, the affinity reached a plateau and gradually decreased in strength, displaying negative cooperativity more typical of dye binding to free DNA. The model proposed by Erard et al. differs significantly from that presented by Wu et al. in that the presence of the histone proteins *reduced* the initial affinity of the dye to the nucleic acid substrate. Positive cooperativity was attributed to the availability of more binding sites through the gradual loosening of the inhibition presented by tight binding of the histone proteins.

How does the presence of chromatin structure formed by the association of histone proteins with DNA alter the affinity and/or the distribution of the dye binding sites on the nucleic acid substrate? In order to evaluate this question, we have undertaken a careful analysis of the binding properties of ethidium molecules to forms of chromatin that represent several levels of DNA folding. We have specifically chosen to begin this analysis using *native* forms of chromatin rather than reconstituted material to avoid any ambiguities that might arise from the reconstitution procedure itself. Previously, we have reported that the binding of ethidium induces a stepwise and time-dependent dissociation of the nucleosome core particle, resulting in the initial loss of one copy each of H2A and H2B (McMurray & van Holde, 1986), a factor not accounted for in earlier binding studies. In this report, we have further characterized the ethidium-induced dissociation process and analyzed the binding properties of ethidium to free DNA and to core particles, accounting for ethidium-induced dissociation. Additionally, we have located the initial binding sites of ethidium on the native core particle structure and compared the dye distribution to that of free DNA. The data are used to construct a model for the binding mechanism of ethidium to chromatin at the level of the nucleosome, integrating the structural consequences of such binding. The model combines some points from the two previous models but differs significantly from both.

Materials and Methods

(*I*) *Preparation of 145 ± 3 bp Native Core Particles.* Highly homogeneous chicken erythrocyte core particles were prepared as described in McMurray and van Holde (1986).

(*II*) *Electrophoresis.* (*A*) *Native Core Particle and Denaturing Gel Electrophoresis.* Native core particle gels and single-stranded denaturing gels were prepared as described in Yager and van Holde (1984).

(*B*) *Protein Gel Electrophoresis.* The protein component of the core particles were analyzed by standard SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) and stained with Coomassie blue.

(*III*) *5′-End Labeling.* Core particles at 9.5×10^{-4} M (bp) (A_{260} = 12.5) were labeled at their 5′-DNA termini by incubation for 1 h at 37 °C with 3.0 μM [γ-^{32}P]ATP (3000 Ci/mmol) (NEN) and 7 units of T4 polynucleotide kinase (BRL, Bethesda, Md.), in a buffer which contained 70 mM Tris, pH = 7.6, 3 mM $MgCl_2$, and 5 mM DTT. The low Mg^{2+} concentration was used to avoid dissociation of the core particles. End-labeled stocks were diluted 1:600 with unlabeled core particles for every dissociation experiment such that the final specific activity was 2 cpm/μL. Core particle complexes were analyzed on nondenaturing 3.5% polyacrylamide gels (McMurray & van Holde, 1986). Sample volumes of 5–15 μL were loaded on the gel and separated, and the gel was exposed to autoradiographic film for 15–24 h. For calibration purposes, a separate 3.5% polyacrylamide gel was developed (simultaneously with each experiment) containing lanes with a series of 5′-end-labeled core particle standards. Each standard was scanned, and the integrated area was used to determine the linear intensity range of the film.

(*IV*) *Laser Densitometry and Quantification of DNA.* DNA or core particle bands were detected by laser scanning densitometry with a Zenieh soft-laser densitometer, Model SL-504-XL, interfaced with an Apple II-C microcomputer. The level of free, dissociated DNA at any input ratio of dye to DNA bp was quantified in two ways: (1) interpolation of sample fluorescence intensities to the fluorescence intensities of DNA standards and (2) quantification of the integrated area from autoradiography of ^{32}P-5′-end-labeled core particles. Both methods have been described in McMurray and van Holde (1986). All measurements were made in the linear intensity range of the film and instrument system used. Integration to determine peak area was carried out by integration software obtained from Biomedical Instruments Software. The integration results were confirmed by cutting and weighing the respective peak areas.

(*V*) *Equilibrium Dialysis.* Equilibrium dialysis experiments were performed in a home-made plexiglass apparatus containing eight separate cells. Each cell contained three (8.4-mL) chambers. In each case, the central chamber was separated from its companion by dialysis membrane. The central chamber of each compartment is the reference chamber and contained the buffer plus the appropriate amount of ethidium. The two outer chambers contained identical samples of nucleic acid so that every individual ethidium/nucleic acid complex was measured in duplicate. The buffer used in all dialysis experiments was 10 mM Tris, pH = 8.0, and 0.1 mM EDTA. The core particle concentration was 3.8×10^{-5} M (bp) (A_{260}

= 0.5). During the entire incubation period, the compartments were sealed so that no loss of volume occurred during the experiment. The samples were allowed to equilibrate for 2–4 days at 30 °C with gentle shaking. After incubation, the concentration of free ethidium bromide was directly determined from the absorbance at 480 nm of samples taken from the central chamber, and the bound ratio of dye was determined as described in the text. Each ethidium/core particle complex taken from the sample cells was analyzed for the level of free, dissociated DNA by quantitative gel electrophoresis.

(*VI*) *Spectrophotometer Measurements.* Absorbance measurements in the UV–visible region were made on a Varian 219 spectrophotometer interfaced to an Apple II microcomputer or on a Varian 2200 spectrophotometer. The temperature in the cell was thermostatically regulated by using a Neslab or Haake circulating water bath and was monitored during the experiment by an internal thermistor. Absorbance measurements were made in 1–10-cm quartz cells, as required, at the appropriate wavelength range for ethidium bromide (λ_{max} = 480 nm). Due to time-dependent dissociation, each titration point was equilibrated for 1 h before the addition of more ethidium. In some cases, the random error was reduced by microcomputing the average of 100 acquired absorbance readings at 480 nm. These averaged absorbance values were converted to bound ratio and free ligand concentration as described in the text. Only data for which the fraction bound ranged between 0.2 and 0.8 were used in the fitting programs.

(*VII*) *Methidiumpropyl-EDTA·Fe(II)* (*MPE*) *Footprint Analysis.* Purified MPE was kindly provided by P. B. Dervan, and MPE footprinting was performed as described in Dervan et al. (1984). Frozen aliquots of MPE (1.0 mg/mL) stock solutions in H_2O were thawed and immediately diluted 1:1 with 1.0×10^{-3} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ solution. The MPE·Fe(II) solution was immediately diluted to 10 mL with 10 mM Tris, pH = 8.0, and 0.1 mM EDTA to yield a final concentration of MPE·Fe(II) = 7.0×10^{-6} M. MPE complexes with DNA or core particles ranged in input ratio of dye to DNA bp (R) from 0 to 0.2 in order to stay within the predissociation binding limit. The MPE/core particle or MPE/DNA complexes (final volume = 3 mL) were made by mixing the components in the following order: buffer, MPE, and nucleic acid stock. Cleavage was initiated by the addition of 30 mM DTT stock solution such that the final concentration of DTT was 3 mM. The cleavage reaction was allowed to proceed for 3 h at room temperature and was stopped with the addition of EDTA to 20 mM. The core particles were deproteinized with Pronase or proteinase K (1 mg/mL) for 1 h at 37 °C, and the core particle DNA was purified by phenol extraction and ethanol precipitation (Maniatis, 1982). The purified DNA was analyzed by native 5–7% polyacrylamide gel electrophoresis (Maniatis, 1975). For some experiments, ^{32}P-5′-end-labeled core particles were added to the MPE reaction. The resulting gels were dried and exposed to autoradiographic film for 16–20 h. DNA bands were quantified by laser densitometry as described above.

Results

(*I*) *Characterization of Ethidium-Induced Dissociation at Low Ionic Strength.* We have previously reported that the binding of ethidium bromide to homogeneous nucleosome core particles results in a stepwise dissociation of the structure which involves the initial release of one copy each of H2A[1] and H2B (McMurray & van Holde, 1986). Under low ionic strength conditions, the dissociation of the core particle is time-dependent and requires that a critical level of dye is bound (a number which we have termed the critical value, ν_c) before

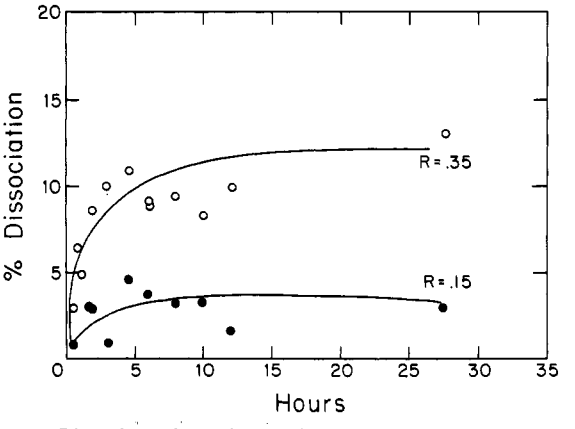

FIGURE 1: Time dependence for ethidium-induced dissociation of the chicken erythrocyte core particle at 30 °C under low ionic strength conditions. (○) Input ratios, R = 0.15; (●) input ratio, R = 0.35. The buffer used in all experiments was 10 mM Tris, pH = 8.0, and 0.1 mM EDTA, and the core particle DNA concentration was $(3.0–7.5) \times 10^{-5}$ M (bp). The core particles were added to solutions of ethidium bromide to yield the indicated ratios of dye to bp. At the indicated times after addition of the dye, the complexes were separated on 3.5% native polyacrylamide, 1.0-mm minigels. The gels were stained, and dissociation was detected by monitoring mobility of free DNA bands. The amount of dissociation was quantified by scanning the negative of the ethidium-stained DNA band with a soft-laser densitometer and comparison of the ethidium-staining intensity to the intensities of a series of DNA standards.

dissociation occurs. In order to determine the kinetics of the dissociation reaction, we quantified the level of free DNA present on polyacrylamide gels (McMurray & van Holde, 1986) at various times after addition of the dye. Figure 1 displays the plots of the percent dissociation versus time at 30 °C for core particle/ethidium complexes at two input dye to bp ratios (R). Under low ionic strength conditions, the overall kinetic rate of dissociation is slow, requiring hours to reach equilibrium. The scatter in the data at low ratios was such that we were unable to determine the kinetic order of the dissociation process. Under low ionic strength conditions, 90% of the dissociation occurs within the first 5 h for all ratios measured. Dissociation is complete in ~25 h.

To demonstrate that the dissociation of the core particles represented an equilibrium process, we measured the extent of reassociation of ethidium-dissociated ^{32}P-5′-end-labeled core particles at 30 °C under low ionic strength conditions. ^{32}P-5′-End-labeled core particles were dissociated by the addition of a high input ratio of ethidium bromide, R = 4.0. After mixing, the sample was allowed to equilibrate at 30 °C for 24 h until dissociation was complete. After equilibration, an aliquot of the R = 4.0 sample was diluted with an equal volume of the intact, unlabeled core particles (R = 0) such that the final concentration of core particles remained constant, but R was reduced by a factor of 2. Successive dilutions of each sample were made until a final ratio of R = 0.03 was reached. The samples were allowed to equilibrate for 24 h and separated on a 3.5% polyacrylamide gel, and the gel was exposed to autoradiographic film for 16–24 h. Figure 2 shows the laser densitometer trace of ^{32}P-end-labeled bands from a representative reversibility experiment. At 30 °C under low ionic strength conditions, successive reduction in the ratio of ethidium results in complete reassociation of the core particles. [A small amount of free DNA is seen in the control (R = 0) lane due to the Mg^{2+} required for the end-labeling process.] The band corresponding to the reassociated core particle is identical in shape and position on polyacrylamide gels with that obtained for intact particles before dissociation. Upon reassociation, the level of free DNA observed in core particle

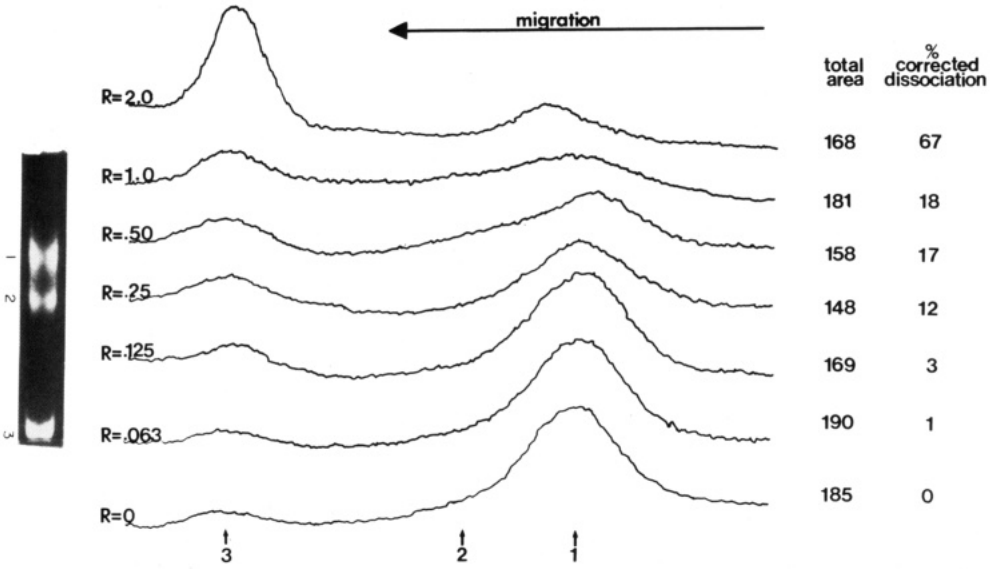

FIGURE 2: Reversibility of the ethidium-induced dissociation of core particles in 10 mM Tris, pH = 8.0 and 0.1 mM EDTA at 30 °C. A core particle/EB complex, $R = 4.0$, was equilibrated for 24 h until dissociation was complete. The sample was successively diluted with equal volumes of intact core particles such that the ratios of dye to bp was reduced by a factor of 2 but the core particle concentration remained constant. A sample of ^{32}P-5′-end-labeled core particles ($R = 0$) was added to each sample. The samples were analyzed by 3.5% polyacrylamide gel electrophoresis after reaching equilibrium, and the dried gels were exposed to autoradiographic film for 20 h. Each lane represents the laser densitometer scan of the autoradiographic film from successive dilutions of the original sample. The arrow at the top of the figure indicates the direction of migration. The gel picture to the left represents the products of the forward dissociation reaction. Arrows labeled 1, 2, and 3 represent the positions of the intact core particle (1), the hexamer particle (2), and free DNA (3) found in the forward reaction. The total area of each peak was integrated and quantified as described under Materials and Methods. The total area of each scan is listed in arbitrary units, and the percent dissociation values were calculated as the ratio of the free DNA band area to the total area. The core particle concentration was 3.8×10^{-5} M.

samples ($R = 0$ to $R = 2.0$) ranges from 0% to 67%, in good agreement with the level of DNA quantified in the forward reaction (McMurray & van Holde, 1986). However, we observed less of the hexamer band intermediate. At all R measured, we observe no loss of signal intensity; i.e., the integrated area of the DNA bands in each lane remains constant (Figure 2). Complete reassociation was observed only at input ratios at or below the critical ratio $\nu_c = 0.05$–0.06 (McMurray & van Holde, 1986). In simultaneous experiments (not shown), to ensure that we were truly measuring reassociation of the end-labeled material and not dilution, we analyzed the identical samples on gels in which the sample volume loaded onto the gel was increased by the amount of dilution. That is, if the original sample was diluted by a factor of 4, then 4 times the sample volume was loaded onto the gel. In all cases, complete reassociation was observed only at input ratios at or below the critical ratio $\nu_c = 0.05$–0.06. We concluded from these experiments that the dissociation of the core particle structure represents a reversible process, induced by the binding of ethidium.

(II) Binding Properties of Ethidium to Free DNA and to the Core Particle at Low Ionic Strength. The absorbance spectrum for ethidium bromide, fully bound either to free DNA or to core particles in 10 mM Tris (pH = 8.0), and 0.1 mM EDTA (TE), exhibits an extinction coefficient of 2300 M^{-1} cm^{-1} at 480 nm, the absorbance maximum. The extinction of the fully bound dye (E_B) is reduced roughly by factor of 2 relative to the extinction coefficient for the free dye ($E_F = 5750$ M^{-1} cm^{-1}) at the same wavelength. The absorbance maximum for both kinds of complex is shifted 40 nm to longer wavelengths (from 480 to 520 nm) relative to the free dye. Spectra obtained on binding ethidium to either free DNA or core particles display well-defined isosbestic points, suggesting that only free and bound states of the dye are present in solution. Assuming that the observed extinction coefficient in the presence of nucleic acid (E_{app}) is a linear function of bound dye, the variation of absorbance at 480 nm as a function of increasing ethidium can be used to calculate the fraction of bound dye at every R. In this type of experiment, the fraction of bound dye (f_B) is calculated by

$$f_B = (E_F - E_{app})/(E_F - E_B) \quad (1)$$

The concentration of bound dye (C_B), the concentration of free dye (C_F), and the ratio of bound dye to DNA base pair (ν) is calculated by

$$C_B = f_B C_T \quad (2)$$

$$C_F = C_T - C_B \quad (3)$$

and

$$\nu = C_B/[D] \quad (4)$$

where C_T is the total concentration of added dye and [D] is the total concentration of DNA measured in base pairs.

The binding results, calculated from the spectrophotometric titration of ethidium to the chicken erythrocyte core particles and to the corresponding free DNA at 30 °C in TE, are shown as Scatchard plots (Scatchard, 1949) in Figure 3A. The binding isotherm for ethidium to free DNA (Figure 3A; circles) displays slight negative cooperativity and a moderate binding constant of $K_A = 1.1 \times 10^6$ M^{-1} in excellent agreement with previous studies (LePecq & Paoletti, 1967; Quadrigfolio et al., 1974; Paoletti et al., 1977; Wu et al., 1980). In contrast, the binding isotherm of ethidium bromide to the core particle (Figure 3A; triangles) is characterized by a positive initial slope. The value for ν reaches a maximum between $R = 0.08$ and $R = 0.10$. At higher values of ν, the curve displays apparent negative cooperativity, resembling that of free DNA.

To ensure the correctness of the highly cooperative binding isotherm for the nucleosome core particle, a second method (equilibrium dialysis) was used to measure binding of ethidium to the core particle, shown in Figure 3B. In the dialysis experiments, C_F is directly measured by the absorbance at 480

Table I: K, n, and ω Computer-Generated Best-Fit Values for the Binding of Ethidium to Free DNA and to Core Particles at 30 °C

sample[a]	K (M^{-1})[b]	n[c]	ω[d]
chicken erythrocyte free DNA (145 ± 3 bp)	1.2×10^6	2.4	0.3
chicken erythrocyte core particle (region A)	2.2×10^4	6.7	140
chicken erythrocyte core particle (region B)	2.7×10^5	2.3	0.4
chicken erythrocyte core particles (corrected for dissociated DNA)	2.4×10^4	4.5	140

[a] All experiments were conducted at 30 °C in 10 mM Tris, pH = 8.0, and 0.1 mM EDTA (TE) buffer. The core particle concentration was 3.8×10^{-5} M (bp) ($A_{260} = 0.5$). Regions A and B refer to different regions of the uncorrected ethidium/core particle binding curve, as described in the text. [b] K (M^{-1}), n, and ω were determined from eq 5 by a nonlinear least-squares analysis program which minimized the sum of the root mean square deviation from the theoretical curve for all experimental points. In this analysis, all three parameters were varied. [c] n is defined as the number of DNA base pairs excluded by the dye. [d] ω is a the cooperativity parameter, defined as the equilibrium constant for transfer of a dye molecule from an isolated site to a singly contiguous site.

nm in the reference chamber. From C_F, the moles of free dye (m_F) is determined by

$$m_F = C_F V_T \quad (5)$$

where V_T is the total volume in the dialysis cell. The number of moles of bound dye (m_B) is obtained by subtracting m_F from the total number of moles added (m_T) to the chamber:

$$m_B = m_T - m_F \quad (6)$$

The value for ν is then

$$\nu = m_B/m_D \quad (7)$$

The equilibrium dialysis results for the binding of ethidium to the chicken erythrocyte core particle are directly compared to the results of the spectrophotometric binding in Figure 3B. Equilibrium dialysis measurements are shown as open circles, and spectrophotometric binding measurements are shown as closed circles. Within experimental error, the results are identical. We note that, independent of the binding method, some traces of aggregation were always observed at very high ν values with time. Thus, for the uncorrected curve, we interpret the x-intercept value for the core particle binding isotherm with caution, and we have avoided quantitative measurements in this region.

The data plotted in Figures 3 were fitted to theoretical expressions for the McGhee and von Hippel (1974) [see also Kowalczykowski et al. (1986)] conditional probability model of excluded site binding. The intrinsic binding constant, K, was determined from the apparent (observed) binding constant, ν/C_F, by using

$$\nu/C_F = K(1 - n\nu)\left[\frac{(2\omega - 1)(1 - n\nu) + (\nu - R)}{2(\omega - 1)(1 - n\nu)}\right] \times (n - 1)\left[\frac{1 - (n + 1)\nu + R}{2(1 - n\nu)}\right]^2 \quad (8)$$

$$R = [\{1 - (n + 1)\nu\}^2 + 4\omega\nu(1 - n\nu)]^{1/2}$$

where n is the number of base pairs excluded to another ligand by each bound dye molecule and ω is the cooperativity parameter which represents the equilibrium constant for the transfer of a dye molecule from an isolated site to a singly contiguous site. The best-fit values for K, n, and ω were calculated from eq 8 by using a nonlinear least-squares fitting program to minimize the sum of the root mean square of the deviation for all experimental points. The results of the K, n, and ω best-fit analyses for ethidium binding to free DNA and to the core particle are listed in Table I. We have chosen to express the fitted parameters only in terms of ligand binding

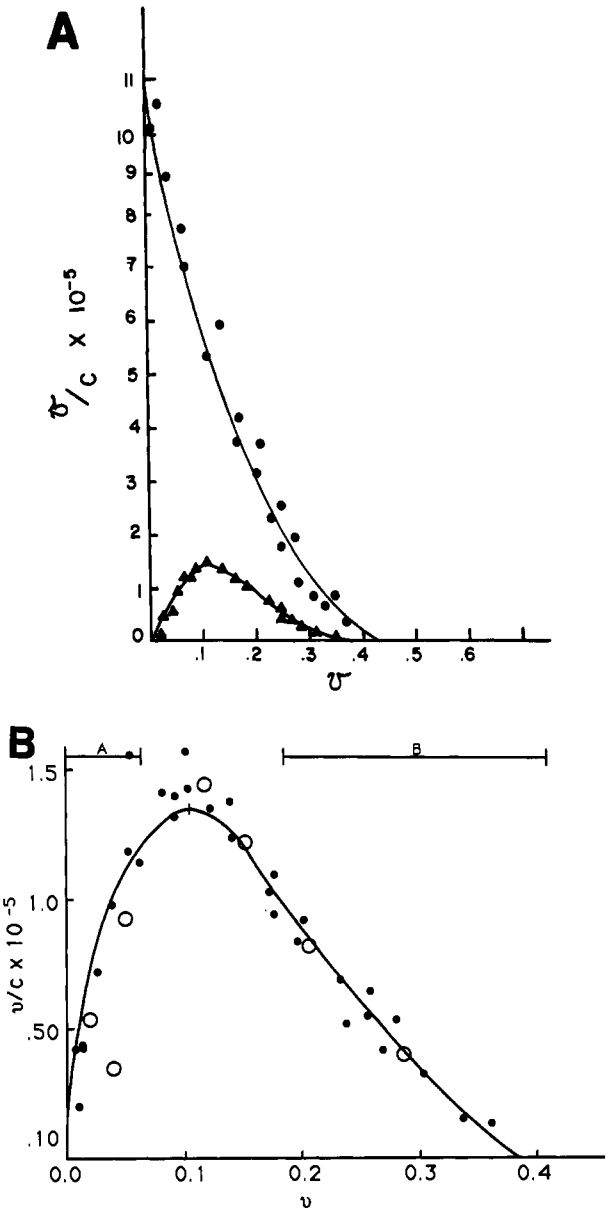

FIGURE 3: (A) Scatchard plot for the binding of ethidium to chicken erythrocyte free DNA and to the chicken erythrocyte core particle at 30 °C: (●) represents the binding curve to free DNA; (▲) represents the binding curve for the core particle. Both curves were generated from the results of a spectrophotometric titration. All experiments were performed in 10 mM Tris, pH = 8.0, and 0.1 mM EDTA at 30 °C. The core particle and free DNA concentration ranged from 1.0×10^{-5} to 7.5×10^{-5} M (bp). (B) Scatchard plot for the binding of ethidium bromide to the chicken erythrocyte core particle at 30 °C measured in two different types of binding experiments: (●) represents the results from the spectrophotometric titration; (○) represents the results from equilibrium dialysis measurements. (A) and (B) indicate the two regions of the ethidium binding isotherm which were fit separately (see text). All experiments were performed in 10 mM Tris, pH = 8.0 and 0.1 mM EDTA at 30 °C. The core particle concentration ranged from 1.0×10^{-5} to 7.5×10^{-5} M (bp).

using the McGhee and von Hippel (1974) model, since this model is not dependent on any specific conformational detail. However, several other models (Dattagupta et al., 1980; Genest & Wahl, 1981; Schmitz 1982) may be used to fit the data. In the model of Dattagupta et al. (1980), one may describe ligand binding in terms of a conformational equilibrium between two states. Results of the best-fit analysis of the data to the allosteric model (Dattagupta et al., 1980; see acknowledgements) yield K_1,K_2 and n_1,n_2 values identical with

the K and n values obtained by applying the McGhee and von Hippel model to regions A and B (see below).

Direct comparison of either the ethidium binding parameters (Table I) or binding isotherms for free DNA and to core particles (Figure 3) reveals major differences in the way in which ethidium binds to DNA in the two states. For free DNA (Figure 3A; filled circles) the binding of the dye is best fit over the entire range by a model with a cooperativity parameter of $\omega = 0.3$, indicating a small degree of negative cooperativity typical of exclusion binding. The maximum affinity for the dye is observed at low values of ν and gradually decreases. In contrast to free DNA, the positive initial slope of the binding isotherm for ethidium bromide to the core particle in TE buffer (Figure 3B) indicates a high degree of *positive* cooperativity; the binding affinity of the dye at very low values of ν is at a minimum. We found it impossible to obtain a good fit to eq 8 for the entire range of core particle binding data using any single set of parameters. Parameters satisfactory at low ν values did not give a good fit at high ν values and vice versa. Consequently, two regions of the ethidium/core particle binding isotherm, indicated in Figure 3B, were analyzed separately. The first region (A) included only the points which constituted the initial positive slope, up to, but not including, the maximum. The second region (B) included only points after the maximum. The K, n, and ω best-fit results for the binding data of ethidium to the core particle in both regions are listed in Table I. Region A points fit well to a theoretical curve characterized by a low intrinsic binding constant, 2.2×10^4 M^{-1}, a large cooperativity parameter, $\omega = 143$, and an $n = 6.7$. The points in region B fit best to a theroetical curve with higher intrinsic affinity, 2.7×10^5 M^{-1}, slight negative cooperativity, $\omega = 0.4$, and an $n = 2.3$.

(III) Correction of Ethidium Binding Curve to the Nucleosome Core Particle for Dissociation. A likely reason why the data shown in Figure 3B cannot be fit by any one set of parameters is the fact that dissociation of nucleosomes occurs above a critical level of bound dye (McMurray & van Holde, 1986). Consequently, the portion of the curve at high ν values must involve considerable ethidium binding to free DNA. To generate the *true* ethidium binding curve for the core particle, we corrected the Scatchard plot shown in Figure 3B for dissociation. The correction involved the determination of the amount of free, dissociated DNA at every input ratio of dye and calculation of the amount of dye bound to the free DNA component, m_B(DNA), and the number of moles of dye bound only to the core particle, m_B(core). The method is schematically diagrammed in Figure 4A. From the uncorrected ethidium binding curve, we calculated C_F and C_B at every input ratio of the dye. Next, the fraction of histone-free DNA arising from ethidium binding was quantified by the gel electrophoretic method described in McMurray and van Holde (1986). From the percent dissociation values, we determined the concentration of free DNA present at every value of ν. Since C_F must be in equilibrium with both the intact core particles and free DNA, the number of moles of ethidium bound to the free DNA component could be calculated directly from the binding isotherm of ethidium to purified free DNA (Figure 3A; filled circles) by

$$m_B(\text{DNA}) = \nu_B(\text{DNA})[\text{D}]V \quad (9)$$

where V is the solution volume. Finally, the m_B(core) was calculated by

$$m_B(\text{core}) = m_B(\text{total}) - m_B(\text{DNA}) \quad (10)$$

Figure 4B shows the graph of percent dissociation versus both

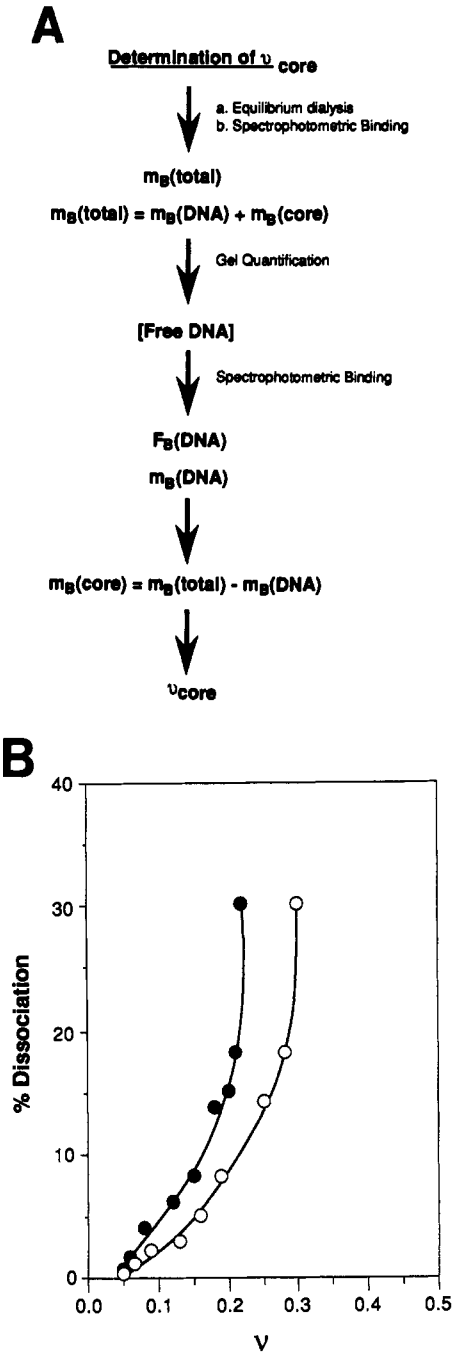

FIGURE 4: (A) Schematic representation of the method used to determine ν_{core}. (B) Plot of percent dissociation versus uncorrected ν values and ν_{core}: (●) represents the percent dissociation versus ν_{core} values; (○) represents the percent dissociation versus the uncorrected ν values. The ν_{core} values were corrected for dissociation by the method described for panel A. The buffer used in all experiments was 10 mM Tris, pH = 8.0, and 0.1 mM EDTA, and the core particle concentration was 3.8×10^{-5} M (bp).

the uncorrected ν values and ν_{core}. Correction of the percent dissociation curve for the amount of ethidium bound only to the core particle results in a dissociation curve which differs significantly only at high ν_{core} values. At low ν values, both the uncorrected and the corrected curve intersect the x-axes near $\nu = 0.05$. At high ν values, the corrected curve asymptotically approaches infinite slope at a limiting ratio at or above $R = 0.20$, differing from the extrapolated value of $R = 0.30$ obtained for the uncorrected curve.

The values of ν_{core} were used to construct a Scatchard plot for the association of ethidium bromide to the core particle, corrected for the presence of free DNA. The results are shown

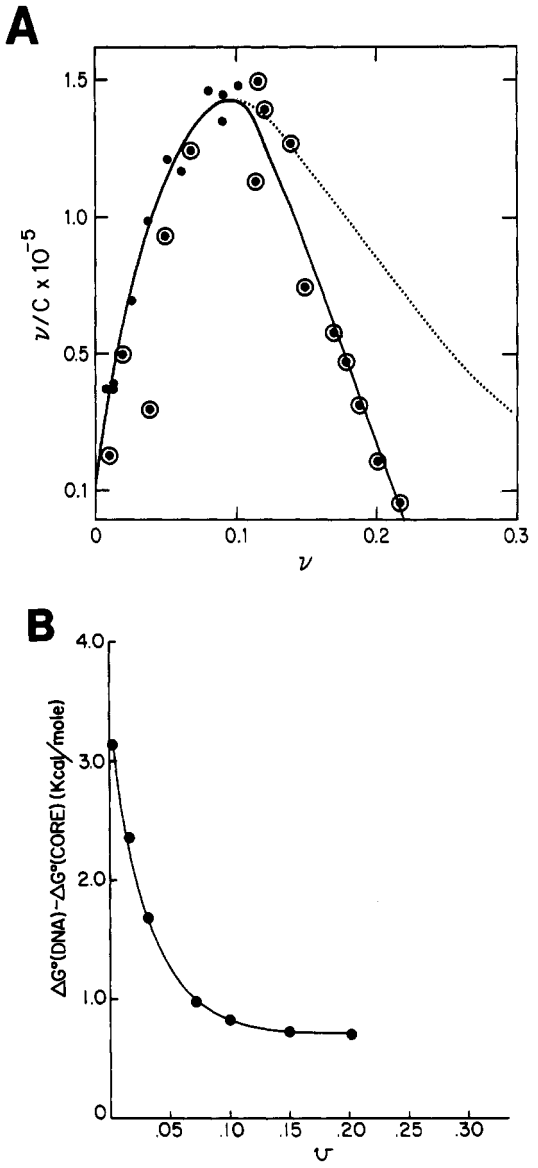

FIGURE 5: (A) Scatchard plot for the binding of ethidium to the nucleosome core particle at 30 °C, corrected for ethidium-induced dissociation. Points represent the results from (⊙) equilibrium dialysis measurements or (●) spectrophotometric measurements. Conditions are as in Figure 4B. The dotted line indicates the uncorrected binding curve. All experiments were performed in 10 mM Tris, pH = 8.0, and 0.1 mM EDTA at 30 °C. The core particle concentration ranged from 1.0×10^{-5} to 7.5×10^{-5} M (bp). (B) Plot of the difference in free energy change between the binding of ethidium to free DNA and to the core protein at 30 °C in TE buffer. The plot was generated by subtracting the $\Delta G°$ values corresponding to the observed binding constants in Figure 3A and panel A of this figure.

in Figure 5A. The binding isotherm of ethidium bromide to the core particle is characterized by a large positive slope which reaches a maximum at $\nu_{core} = 0.08$–0.10. The initial positive slope in the binding curve corresponds to the region in the binding curve where we detect *no* dissociation to free DNA. The binding isotherm for the corrected curve was also fit to eq 8. For the corrected curve, we were able to obtain good fitting over the entire range to a single set of parameters. The results of the fitting analysis are listed in Table I. The corrected binding curve displayed a small intrinsic binding constant of $K_A = 2.4 \times 10^4$ M^{-1}, a cooperativity parameter of $\omega = 140$, and an n value of 5.0. Comparison of the best-fit K, n, and ω values for the dissociation-corrected binding curve to region A points of the uncorrected curve reveals that the two isotherms are almost superimposable. Thus, only the initial points of the uncorrected ethidium/core particle binding curve actually represent binding to the core particle. We conclude that the rest of the uncorrected curve represents ethidium binding to dissociated DNA. This conclusion is supported by the fact that correction of the binding curve for ethidium bound to free DNA abolishes the entire tail of the uncorrected binding curve. Furthermore, theoretical fitting of K, n, and ω for region B points in the uncorrected curve were in approximate agreement with the K, n, and ω values observed for ethidium binding to free DNA.

Comparison of the binding parameters for ethidium to free DNA (Figure 3A) and to the core particle (Figure 5A) reveals that the binding affinity of the first ethidium molecule for DNA is roughly 100-fold less when that DNA is folded into a core particle structure. The free energy difference between the binding of ethidium to free DNA and to the core particle, calculated from the observed binding constants (ν/C), is plotted in Figure 5B. At the very lowest ratio, the binding of ethidium to free DNA is favored by 3.5 kcal/mol over binding to the core particle. As the level of dye is increased, the energy difference of ethidium binding to the two forms decreases until it essentially remains constant at 0.8 kcal/mol near a ν of 0.1. Thus, on the basis of their respective binding constants, the first ethidium molecules will prefer to bind free DNA sites relative to core particle DNA sites with a probability ratio of 83:1 if equal populations of both are present.

(IV) Location of Ethidium Binding Sites on the Nucleosome Core Particle. In addition to the large differences in binding constant and cooperativity between the two forms of nucleic acid, comparison of the values for the n parameter in Table I indicates that the number of base pairs apparently excluded by each molecule of dye is much larger when the dye is bound to the core particle ($n = 6.0 \pm 1$) than when it is bound to free, random sequence DNA ($n = 2.4$). In the McGhee and von Hippel analysis, the value of n is obtained as the reciprocal of the x-intercept value at saturating levels of ligand, with the assumption that, at least initially, the entire lattice is available for binding. For the core particle, the possibility that the histones presented an obstacle to dye binding at some points along the DNA lattice led to the consideration of two binding models which could both account for the larger n observation. The first possibility is that the dye molecules could bind randomly over the entire lattice, an average of 1 every 5 base pairs. The second possibility might be called regional binding. In this case, only particular region(s) on the lattice is (are) actually available for dye binding due to the presence of histones. If only a fraction of lattice is available for binding, even if the dye binds randomly within this regional limit, then the apparent value for n will be larger than the true value.

To distinguish between these two cases, we devised the following experiment, which is schematically illustrated in Figure 6. We monitored the binding of an analogue of ethidium bromide, methidiumpropyl-EDTA·Fe(II) (MPE) (Hertzberg & Dervan, 1982), to 5′-^{32}P-end-labeled core particles primarily within the *predissociation* range of input ratios. MPE has binding properties quite similar to ethidium but contains the metal chelator, EDTA, tethered to the intercalator portion of the molecule. Upon activation with agents such as dithiothreitol (DTT), MPE cleaves one strand of the DNA at or very close to the binding site of the dye molecule. The complementary strand can then be cut with S1 nuclease to yield a double-stranded cut. Since the length of the DNA in the core particle is 145 bp, we predicted that random binding of MPE should result in the immediate breakdown of the DNA

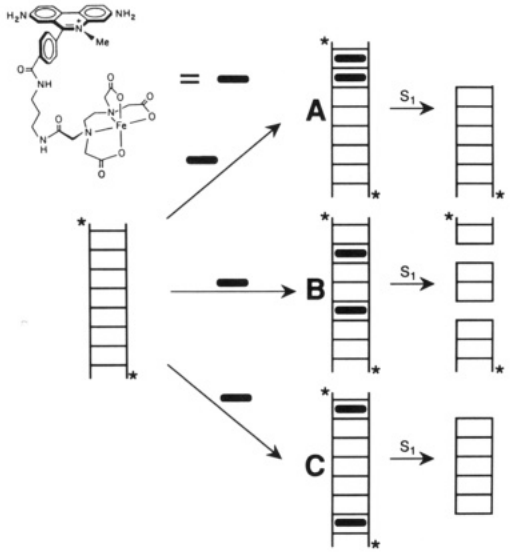

FIGURE 6: Schematic experimental design for determination of the location of ethidium binding sites on the nucleosome core particle. The structure at the top is methidiumpropyl-EDTA·Fe(II), an ethidium analogue that can cleave the DNA at its binding site. The ladders represent the DNA lattice, and the stars represent ^{32}P-5′-end-labeled termini. S1 indicates endonucleolytic cleavage by S1 at MPE-induced single-stranded breaks in the phosphodiester backbone. (A) represents predicted results for regional MPE binding at a single DNA terminus; (B) represents predicted results for random binding of MPE anywhere on the DNA lattice; (C) represents predicted results for regional MPE binding at both DNA termini.

into a series of small oligonucleotides as shown in Figure 6B. Since the sample of core particles contains random sequences of DNA, we should observe, after ethidium staining, a smear of fluorescence intensity with the eventual disappearance of the DNA band at high ratios, i.e., when the cleavage products are very small. On the other hand, if the binding were regional, we should observe discrete DNA bands whose length should indicate the region of dye binding. Figure 6A,C illustrates predicted results of regional binding to one (A) or both ends (C) of the core particle. The core particles were complexed with MPE, activated with DTT, and deproteinized to remove the histones. The resulting purified DNA was digested with S1 nuclease to create a double-stranded cut, and finally, the DNA fragments were separated by 7% polyacrylamide gel electrophoresis. Analyses of the cleavage products of the MPE reaction were utilized to determine the binding site of the dye and to distinguish between random versus regional binding.

The results of the MPE cleavage reaction of free DNA (lanes 1–4) and the core particles (lanes 5–10) are shown in Figure 7. Two lanes in each case are $R = 0$ control lanes: lanes 1 and 2 for free DNA and lanes 5 and 6 for the core particles. The DNA in lanes 1 and 5 were directly extracted from the intact core particles or DNA without exposure to any of the reaction conditions. This control will be referred to as the untreated control lane. Lanes 2 and 6 were mock control reaction; i.e., the DNA went through all the reaction steps minus the MPE. For both free DNA and the core particle, the amount of DNA detected in the mock control was always less than the untreated control. Thus, some amount of nucleic acid/MPE complex was always lost during the reaction conditions and purification procedure. Consequently, the integrated area in the presence of dye is always compared to the mock experiment. At $R = 0.02$ (lane 3, Figure 7A), we observe immediate degradation of the free DNA indicated by loss of intensity of the intact band. At $R = 0.06$ (lane 4, Figure 7A), all the DNA was cleaved into small oligonucleotides and, as expected, no DNA can be detected on the gel. Thus, for

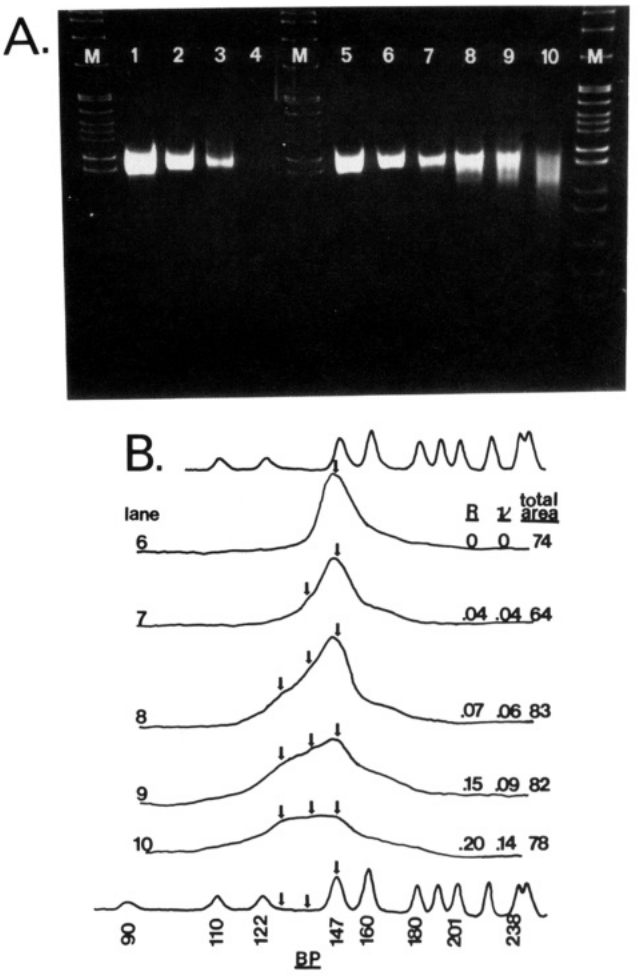

FIGURE 7: Results of the MPE cleavage reaction with the nucleosome core particle and the corresponding free DNA. (A) Gel electrophoresis of the MPE cleavage products generated from MPE/DNA complexes and MPE/core particle complexes. Lanes: (1) free DNA, $R = 0$; (2) free DNA, $R = 0$, mock; (3) free DNA, $R = 0.02$; (4) free DNA, $R = 0.06$; (M) DNA molecular weight standards generated from a *Hpa*II digest of PBr322; (5) core particle, $R = 0$; (6) core particle, $R = 0$, mock; (7) core particle, $R = 0.02$; core particle, $R = 0.06$; core particle, $R = 0.10$; (8) core particle, $R = 0.20$. (B) Laser densitometer trace of the bands seen in (A). The lengths of the DNA molecular weight markers are indicated. Arrows indicate the presence of peaks for a predominant cleavage product distributions. The bands were analyzed by 5% native polyacrylamide gel electrophoresis.

free DNA, the results from MPE cleavage clearly indicates random binding of the dye. In contrast, MPE cleavage of the core particles results in the appearance of discrete bands which are shorter than those for the mock control (lane 6, Figures 7A and 8A). The laser densitometer traces of the core particle DNA bands are shown in Figure 7B. Increasing input ratios of MPE to core particle DNA (from 0.02 to 0.20) results in the loss of intensity in the region near 145 bp with a concomitant increase in intensity in the region between 145 and 120 bp. The intensity appears to be distributed in at least three peaks: one near the intact band (140–142 bp), one centered at 135 bp, and one centered at 120 bp. Thus, even the longest DNA band appears to be shortened by a few bp. At the highest input ratio used ($R = 0.20$), a small amount of material smaller than 120 bp appears; this appearance coincides with the beginning of particle dissociation. Over the entire ratio range, the total intensity in all lanes remains constant. Thus, MPE cleavage of the DNA within the intact core particle results in the initial loss of up to 25 bp. Since fragments of length = 120 bp could not be generated by cutting within the center of the core particle, the cleavage pattern demonstrates

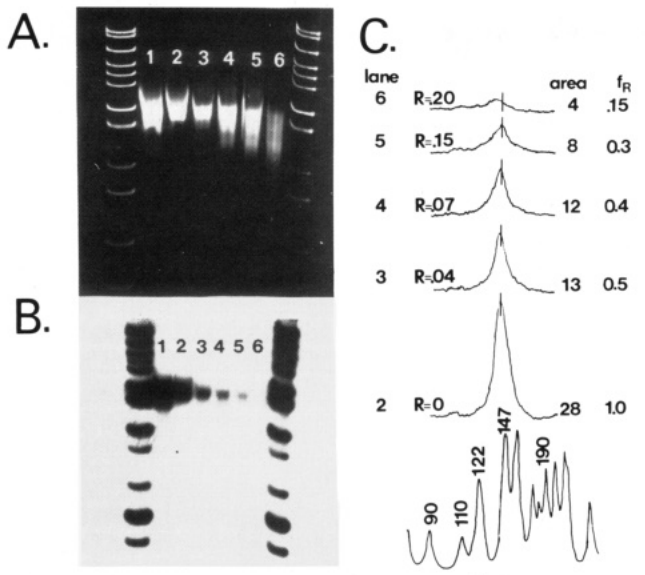

FIGURE 8: Results of the MPE cleavage reaction of ^{32}P-5′-end-labeled core particles. (A) Results of the MPE cleavage reaction visualized by ethidium staining of a 7% native polyacrylamide gels: DNA molecular weight markers generated by a *Hpa*II digest of PBr322; (1) core particles, $R = 0$; (2) core particles, $R = 0$, mock; (3) core particles, $R = 0.02$; (4) core particles, $R = 0.06$; (5) core particles, $R = 0.10$; (6) core particles, $R = 0.20$. (B) Results of the autoradiography of ^{32}P-5′-end-labeled core particles in (A). (C) Laser densitometer trace of the bands in (B). The lengths of the DNA molecular weight markers are indicated.

that, under nondissociating conditions, the ethidium binding sites on the core particle occur only at the ends of the core particle.

While fragment lengths, generated by the MPE cleavage reaction, unambiguously identified the core particle DNA termini as the site of binding for MPE, the end-binding reaction can occur by two different mechanisms. First, the dye could bind to 25 bp located at a single DNA terminus. Alternatively, the MPE molecules could bind to both ends. In this case, we would expect that dye binding would cleave a total of 25 bp of DNA, distributed in some manner at both termini. These mechanisms are schematically depicted in Figure 6A,C. To elucidate which of these two possibilities is correct, we monitored the fate during an MPE cleavage reaction of the terminal 5′-^{32}P-label attached to both ends of the core particle. Dye binding to only one end would be confirmed if the intensity of the band was reduced by half, due to the cleavage and loss of one ^{32}P-5′-end, *concomitant* with a reduction in the length of the end-labeled fragment. On the other hand, if dye binding occurs at both ends, cleavage of both ^{32}P-5′-termini would prevent detection of these bands on an autoradiograph. In this case, we would expect to observe gradual disappearance of band intensity, accompanied by little to no reduction in fragment length. The results of these experiments, shown in Figure 8, confirm that dye binding occurs to both ends of most of the core particles. At $R = 0$ (Figure 8B, lane 2), all the intensity resides in the 145 bp band of the intact deproteinized core particle DNA. As the input ratio of dye to base pair is increased from $R = 0.04$ to $R = 0.20$ (Figure 8B,C, lanes 3–6), we observed a gradual reduction in band intensity concomitant with little reduction in band length. Above an input ratio of $R = 0.20$ (bound ratio of 0.12), only 14% of the intensity corresponding to ^{32}P-5′-end-labeled core particle DNA remained, indicating that both the 5′-DNA termini were missing from most particles. Comparison of ethidium staining intensity (Figure 8A) and autoradiography film intensity (Figure 8B) reveals that, in the same ratio range where the DNA intensity had virtually vanished from the autoradiographic film, the integrated area of the ethidium staining intensity was nearly equal to that of the $R = 0$, uncleaved DNA band (lanes 2 and 6, Figure 8A). Thus, between $R = 0.04$ and $R = 0.20$, essentially all the DNA was redistributed into DNA species of smaller than 145 bp in length which were missing both their DNA termini.

(*V*) *Distribution of Binding among Nucleosomes. Determination of f.* The ^{32}P-end label results shown in Figure 8B,C were also used to determine f_R, the fraction of core particle ends that remain after MPE cleavage. These data were used to estimate the fraction (f) of core particles which actually bind dye before dissociation begins (McMurray & van Holde, 1986). We had initially estimated ν_c under the assumption that $f = 1.0$, i.e., that all core particles bound dye below the critical value. However, the highly positive cooperativity, characteristic of ethidium binding to the core particle, indicated that f might be less than 1.0. That is, at low input ratios, those core particles that initially accepted dye molecules would have an enormous advantage in accepting more, as compared to others with no bound dye. Therefore, we directly measured the f from the results of the MPE experiment. Since most of the core particles bind MPE from both ends, MPE binding and cleavage of a ^{32}P-end-labeled core particle results in the disappearance of band intensity detected by autoradiography. Thus, only core particles that do *not* bind dye or that bind very little dye will be visible on the autoradiogram, and f can be measured from the fractional band intensity remaining (f_R) after cleavage with MPE at the critical value of $f = 1 - f_R$). The f_R value at each input ratio is listed in Figure 8C. The critical *bound* ratio, $\nu = 0.05$, corresponds to an *input* ratio close to $R = 0.06$. Thus, the minimum value for f_R and f is approximately 0.5. Since $f < 1.0$, then the correct critical ratio is ν_c/f (McMurray & van Holde, 1986). We conclude that a maximum value of ν_c must be at 0.10; i.e., an average of 14 ethidium molecules are required for dissociation. This value involves the assumption that all of the apparent "full-sized" molecules revealed by autoradiography in Figure 8 have both ends intact. Whatever the quantitative value of f may be, the fact that $f < 1.0$ reveals an important new aspect of the dissociation mechanism. At low ionic strength, dissociation is also a highly cooperative process with some core particles dissociating before others bind any dye at all.

Discussion

We are utilizing the binding of a nonspecific intercalator, ethidium, in order to construct a model that predicts the binding frequency of ethidium to various chromatin states inside the cell. In order to construct such a model, it is necessary to have a detailed understanding of the mechanism and effects of ethidium binding at every level of DNA folding. At the level of the nucleosome core particle, previous reports disagreed on both the mechanism and effects of ethidium binding. We have previously reported that the binding of ethidium induces disssociation of histone proteins (McMurray & van Holde, 1986). In continuation of these studies, we have carefully examined the association of ethidium to the nucleosome core particle under low ionic strength conditions, taking account of ethidium-induced dissociation. It is important to note that dissociation is induced by *intercalation* binding of ethidium to core particle DNA and is not induced by ethidium binding to histones or by nonintercalative ethidium binding modes, as shown in the following paper in this issue (McMurray et al., 1991). The corrected binding curve reveals that dye binding to the core particle is a highly cooperative process, $\omega = 140$, characterized by a low intrinsic binding

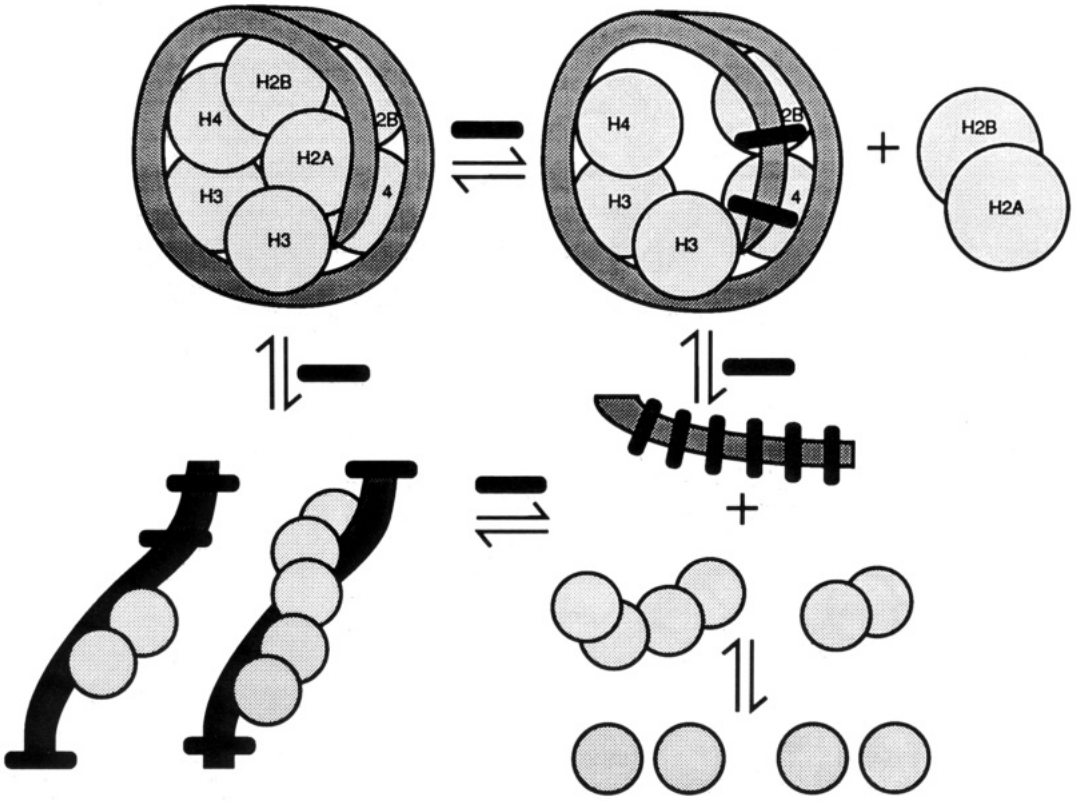

FIGURE 9: Schematic representation of the model for ethidium binding to chromatin at the level of the nucleosome core particle and ethidium-induced dissociation of the core particle structure.

affinity, $K_A = 2.4 \times 10^4\ M^{-1}$. Comparison of the intrinsic binding constants between ethidium binding to the core particle and free DNA reveals that, for the first few ethidium molecules, binding affinity to free DNA is roughly 100-fold stronger than binding to DNA in core particles; in terms of free energy it is favored by 3.5 kcal/mol. As the level of ethidium increases, binding will be increasingly favored to the dissociated DNA. The neighbor exclusion principle dictates that complete saturation of a free DNA lattice with ethidium will occur at $\nu = 0.5$ (McGhee & von Hippel, 1974; Crothers, 1968). Correction of the ethidium binding curve for the amount of dye bound to dissociated free DNA indicates that only the region A points, i.e., those points that occur before the cooperative maximum, truly measured binding of ethidium to the core particle. The rest of the uncorrected binding curve represents, almost entirely, ethidium binding to free, dissociated DNA.

MPE footprinting analysis reveals that the initial ethidium binding sites were not distributed in a random fashion around the core particle but instead were clustered near the DNA termini. Thus, the association of the histone proteins to the free DNA to form the core particle structure alters the distribution of dye binding sites on the DNA lattice. Approximately 25 bp of DNA were involved with the end-binding reaction of MPE, distributed in some manner at both ends of the core particle. Regional binding of ethidium to the ends of the core particle DNA explains the large apparent exclusion value observed for ethidium when bound to the core particle ($n = 5$–7) relative to free DNA ($n = 2.4$). Both binding and dissociation approached a limiting ratio at $\nu = 0.20$. Thus, the dissociation curve indicates that ~29 bp of core particle DNA are theoretically available for dye binding before dissociation on the intact core particle if all the core particles bind dye ($f = 1.0$). MPE footprinting showed that as R increases to $R = 0.15$, the fractional population of core particles that bind dye (f) increases from 0.5 to 1.0. Thus, the steep slope of the dissociation curve represents an increasing fraction of core particles which have bound the critical level of dye for dissociation. We begin to see dissociation at the lower limit of the curve, representing the critical value when about half of the particles bind dye. The upper limit of the dissociation curve represents the critical value when f approaches 1.0. Estimation of the critical value from MPE footprinting analysis (20–25 bp of DNA) and the limiting ratio of the dissociation curve, $\nu = 0.20$ (29 bp of DNA), were in excellent agreement, indicating that an average of ~27 ± 4 bp of core particle DNA are bound to dye prior to disruption of particle structure. Thus, after a core particle binds dye to 27 ± 4 bp of DNA (roughly 14 ethidium molecules), the particle will dissociate. We propose the following model of ethidium binding to the core particle and ethidium-induced dissociation, schematically represented in Figure 9:

(*1*) *Dye Binding to the Core Particle DNA Is Initially an Unfavorable Event.* The core particle, under a range of moderate temperature and ionic strength conditions, appears to be a highly stable complex (Yager et al., 1989; Yager & van Holde, 1984; Ausio et al., 1984; Bina et al., 1980; Weischet et al., 1978). The low intrinsic binding constant of ethidium to the core particle structure suggests that, at least at very low ratios, the ethidium molecule is structurally inhibited from binding to the core particle. The unfavorable interaction of the dye with intercalation site probably arises from a combination of conditions. First, the base pair opening motions within the helix may be suppressed due to histone association (McMurray et al., 1985). The high intrinsic fluorescence anisotropy of ethidium bound to the core particle (Paoletti et al., 1977; Hurley et al., 1982) suggests that the internal flexibility of the core particle DNA is decreased relative to free DNA. Since it is likely that the binding of ethidium requires an opening motion of the DNA base pairs to expose

the intercalation site, a reduction in the open/closed equilibrium constant may decrease the availability of binding sites. We predict that the initial binding event will display slower kinetics than initial binding to free DNA. Second, since ethidium binding has an electrostatic component to binding free energy, it may compete for binding sites with the histone proteins. Thus, the presence of the histones may present a steric block to the binding of dye molecules. Finally, since the binding of dye molecules destabilizes the core particle structure, the free energy of binding must offset any loss of stability of the core particle structure due to dye binding. For all these reasons, we might expect the initial dye binding event to be thermodynamically unfavorable.

(2) *The Sites of Initial Dye Binding Are Located Exclusively at the Ends of the Core Particle DNA Termini.* As the concentration of ethidium molecules increases, the first dye molecule will eventually bind. MPE footprint analysis reveals that the available sites for initial binding are located exclusively at the termini of the core particle DNA. End binding of the ethidium molecules might be expected as the favored reaction since the ends of the core particle are stabilized by fewer histone contact points in these regions (Morse & Simpson, 1988). Chemical cross-linking experiments (Schick et al., 1980) and thermal melting studies (Weishet et al., 1978; Simpson, 1979) have all indicated that ~20 bp at each end of the core particle, a total of 40 bp, are stabilized by a lower density of histone contact points relative to the interior of the core particle DNA. The MPE cleavage experiments and determination of ν_C from the dissociation curve have indicated that an average of 27 bp of DNA are involved in the predissociation binding of ethidium. Additionally, MPE cleavage of ^{32}P-5′-end-labeled core particles revealed that dye binding occurs at *both* ends of the core particle DNA termini.

(3) *Ethidium Binding Results in the Initial Release of One Copy Each of H2A and H2B. Dye Binding to the Core Particle Is an Asymmetric, Positive Cooperative Process Resulting from the Appearance of New DNA Binding Sites Due to Histone Loss.* The binding of ethidium to the nucleosome core particle induces a stepwise dissociation of the core particle structure, resulting in the initial release of *one* copy each of H2A and H2B (McMurray & van Holde, 1986). Since positive cooperativity is not observed for dye binding to free DNA, the positive cooperativity characteristic of the ethidium binding to the core particle arises, in part, from the appearance of initially inhibited DNA binding sites, due to loss of histones. While the binding of ethidium results in the initial loss of only *one* copy each of H2A and H2B prior to complete dissociation of the DNA, MPE binding analysis indicates that initially *both* ends of the core particle bind ethidium. Thus, double-end binding of ethidium results in the loss of proteins on only one side of the particle. Two possible models fit the data. First, dye binding occurs cooperatively and to an equal extent at both ends of the core particle. However, ethidium binding induces random sliding and/or reorientation of the of the histone octamer such that one copy of H2A and H2B remains in contact with DNA while the other is lost, prior to dissociation. While this possible model cannot be ruled out on the basis of the data, we might expect that if this model were true, then we would occasionally observe a tetramer particle, that is, a 145 ± 3 bp length of DNA complexed with only two copies each of H3 and H4. Additionally, any reorientation of the histone core proteins that would protect one of the H2A–H2B dimers would tend to expose H4. Thus, we also might expect to observe an intermediate containing depleted amounts of H4. Neither of these types of intermediates has ever been observed in our experiments. For this reason, we favor the remaining model of asymmetric binding. In this model, the ends of the core particle are initially equivalent binding sites for dye, and both ends are capable of binding a few ethidium molecules. However, dye binding loosens the histone association, and once histones are loosened, a critical level of dye binding occurs randomly to one end or to the other. In this model, we would expect that the level of bound dye per DNA terminus of the core particle is asymmetric. Binding continues to the preferred end until one H2A and H2B pair is released; further dye binding leads to complete dissociation of the core particles. Thus, the first random end-binding events and subsequent loosening of histones dictate some preferred direction to further binding. The directional binding of ethidium explains the loss of only one copy each of H2A and H2B, giving rise to the hexamer particle. The idea of directional binding is supported by the electron microscopy data of Erard et al. (1979). The authors observed that intact core particles, uncomplexed with dye, displayed a roughly spherical shape of diameter 140 ± 15 Å. In contrast, core particle/ethidium complexes, when visualized by electron microscopy, were roughly spherical but exhibited a single DNA tail of variable length. No particles with two tails were observed. Consistent with the highly cooperative binding, our MPE footprinting analysis revealed that, prior to dissociation, roughly half of the core particles actually bind the dye. Therefore, below the critical ratio, at least two populations of core particles must exist in solution, one intact and uncomplexed with dye while the other has bound dye and may have lost a portion of its complement of histone proteins.

(4) *Further Binding of Ethidium to the Hexamer Particle Results in Complete Dissociation of the Particle to Free DNA and Histones.* Once ethidium has gained entry, the geometry of the compact particle is disrupted, resulting in the complete dissociation of the core particle to free DNA and histones. Since ethidium binding lengthens and stiffens the DNA helix (Lerman, 1961, 1963, 1964), we conclude that dissociation occurs because the DNA complexed with ethidium is no longer flexible enough to fold around the histone protein core. Additionally, the ethidium cation neutralizes some of the negative charge on the DNA phosphate backbone, reducing the number of potential electrostatic interaction sites with the histone Lys/Arg residues. Both cross-linking studies (Schick et al., 1980) and X-ray crystallographic data (Richmond et al., 1984) indicate that the binding site of one H2A and H2B dimer is ~25 bp long. Thus, binding of the critical level of dye correlates with the exclusion of one copy each of H2A and H2B. Since only the innermost histones, H3 and H4, are required for folding the particle (Read et al., 1985; Camerini-Otero & Felsenfeld, 1977), dissociation occurs only after the loss of H2A and H2B because, at this point, the continued binding of dye molecules begins to perturb H3,H4 interactions.

(5) *Ethidium-Induced Dissociation Is Completely Reversible.* Removal of ethidium bromide resulted in complete reassociation of the histones with DNA to form intact core particles.

Comparison with Other Data and Conclusion. Our model for ethidium binding resolves several discrepancies in earlier studies of ethidium/nucleosome interactions. First, ethidium binding to the core particle is a positive cooperative process, confirming the results of Erard et al., (1979). At present, we cannot explain the difference in binding isotherms between ours [and Erard et al. (1979)] and that obtained by Wu et al. (1980). One possibility is that the high binding affinity

and negative cooperative curves observed by Wu et al. (1980) for ethidium binding to nucleosomes were the result of heterogeneity in the sample preparation. Comparison of the ethidium binding curves for free DNA and for core particles from our results clearly shows that the binding to these two forms of nucleic acid differ dramatically in both affinity and cooperativity. Thus, preparations containing significant amounts of dissociated DNA or nucleosomes that contain portions of linker DNA could give rise to binding isotherms similar to that reported by Wu et al. (1980). It is interesting to note that an ethidium binding isotherm reported in subsequent studies (Chaires et al., 1983) showed a superimposable binding curve using 175 bp nucleosomes. Thus, studies from the same laboratory indicated no difference in ethidium binding to core particle preparations containing 140 bp of histone-associated DNA (Wu et al., 1980) and 175 bp nucleosome which contained 140 bp of histone-associated DNA and 35 bp of free, linker DNA (Chaires, 1983). It is also important to note that the nucleosomes used in the studies of Wu et al. (1980) and Chaires et al. (1983) were isolated from calf thymus rather than chicken erythrocytes. Since the amino acid sequences of histones from these two sources differ slightly, it is also possible that nucleosomes isolated from the two sources behave differently. The full explanation for the affinity and cooperativity differences in ethidium binding will await further study.

Next, we find that the binding of ethidium to the core particle above the critical value clearly results in dissociation of histones. Wu et al. (1980), using SDS–protein gel electrophoresis of sucrose gradient fractions (Laemmli, 1970), reported that all four histones were present in ethidium/nucleosome complexes below $R = 0.1$ and concluded from these results that histone association was maintained during dye binding. However, we have shown that *at least* two populations of core particles exist at the lowest ratios of dye to bp, intact cores, and hexamers. Subsequent to the formation of the hexamer, ethidium induces a complete dissociation of free DNA (McMurray & van Holde, 1986). Thus, even in the presence of ethidium, some fraction of the population of core particles will remain intact and will retain their full complement of histones. Detection of proteins by gel electrophoresis of sucrose gradient fractions for ethidium/nucleosome complexes at low ratios may be insensitive to a small fraction of hexameric particles in the presence of whole nucleosomes.

Although we reproduced the positive cooperativity observed by Erard et al. (1979), several aspects of ethidium binding to the core particles observed in their work have been clarified or redefined. First, we find that the degree of positive cooperativity is much greater than these authors estimated. We find that our best fit to the data indicates an $\omega = 140$, while they estimated an $\omega = 24$. Second, the authors did not detect histone dissociation and therefore did not correct the binding isotherms for ethidium-induced dissociation of the DNA. Instead, they proposed that intercalation did not occur above $R = 0.1$ and that the negative cooperative curve observed after the cooperative maximum was due to the purely electrostatic binding of dye molecules. Both Wu et al. (1980) and Erard et al. (1979) propose that unfolding of the particle structure occurs rather than dissociation of histone proteins. We find that, while a small change in radius (2–5 Å) does occur prior to dissociation, the presence of multiple forms of ethidium/nucleic acid complexes induced by stepwise dissociation of the core particle (the intact octamer, the hexamer, and free DNA) (McMurray & van Holde, 1986) accounts for most of the observed "unfolding" of the core particle structure. Finally, Erard et al. (1979) observed that half the core particle/ethidium complexes, when visualized by electron microscopy, were found to be unfolded. The authors ascribed this heterogeneity to differential micrococcal nuclease digests of nucleosomes which gave rise to two subpopulations of core particles. We have shown that only a fractional population actually binds dye initially and that this population increases with increasing ratio of ethidium to DNA bp. That a fraction of core particles exists that does not bind dye after ethidium is added is purely a thermodynamic consequence of the large degree of positive cooperativity characteristic of dye binding to the core particle DNA.

Finally, our results have implications that may relate to the biological consequences of ethidium binding in vivo. First, we have established that the association of histone proteins with free DNA to form the core particle structure clearly *reduces* the affinity of dye binding for its nucleic acid substrate. Thus, ethidium binding to chromatin, at the level of the nucleosome, is not a favored reaction, especially in the presence of any free DNA substrate. The binding of MPE, an ethidium derivative which binds similarly to ethidium yet cleaves the DNA at or near the dye binding site, has indicated that the internucleosomal linker DNA is the preferred binding site for the dye within genes, when bound in vivo (Cartwright et al., 1983; Benezra et al., 1985). Thus our results give a firm thermodynamic basis for the observed in vivo linker preference and distribution of MPE. If ethidium is forced to bind extensively within the core particle regions, the dye will bind at the expense of histone proteins and nucleosomes structure will be destroyed.

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