Intermolecular Histone H4 Interactions in Core Nucleosomes†

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ABSTRACT: Chicken histone H4, labeled at methionine-84 with 1-*N*-pyrenyliodoacetamide, has been incorporated into a nucleosome-like particle with core length DNA and unmodified histones H2A, H2B, and H3. These synthetic nucleosomes exhibit properties very similar to those displayed by native particles and those labeled with other fluors. The emission spectrum of the pyrene-labeled nucleosome was characteristic of excited dimer (excimer) fluorescence, indicating that the single pyrene groups on the two H4 molecules are in close proximity in the reconstituted particle. Histone H4 was also labeled randomly at lysines with a group that contains two pyrene moieties separated by 12 Å at most. Incorporation of this histone into nucleosome-like particles provides an excimer standard which does not depend on intermolecular interactions. The properties of the pyrene-containing nucleosome were examined as a function of ionic strength. It was found that the H4–H4 pyrene excimer fluorescence exhibited a cooperative disruption centered at 0.1 M NaCl which preceded increases in accessibility and environment polarity revealed by other fluors attached at the same site.

In recent X-ray diffraction studies of the core nucleosome at 7- and 15-Å resolution, not all histone–histone interfaces could be assigned with certainty (Richmond et al., 1984; Uberbacher & Bunick, 1985). Knowledge of the precise arrangement of the eight histone molecules within the core nucleosome is an essential prerequisite for understanding the molecular basis of structural changes such as those associated with the DNase I sensitivity of transcriptionally active genes. Certain inter-histone contacts such as H3–H3 and H3–H4 have been incorporated into the model for the current structure from the results of cross-linking and fluorescence experiments (Klug et al., 1980). Evidence for a major H3–H3 contact comes from the ability of a histone H3–H3 disulfide-linked dimer to be incorporated into nucleosome-like particles (Camerini-Otero & Felsenfeld, 1977; Lewis & Chiu, 1980) and from fluorescence studies utilizing extrinsic fluors covalently bound to the single Cys-110 residue in chicken histone H3 (Dieterich et al., 1977, 1979; Prior et al., 1980; Daban & Cantor, 1982a,b).

In spite of a theoretical paper predicting an H4–H4 contact in the nucleosome (Ohlenbush, 1981) and the fact that this histone exhibits specific intermolecular interactions in free solution (D'Anna & Isenberg, 1974; Lewis et al., 1975), no evidence has heretofore been obtained to support the existence of this intermolecular contact. We report here the results of experiments with histone H4 labeled with pyrene at Met-84 which indicate the existence of a histone H4–H4 contact in the vicinity of the bound fluor when this histone is incorporated by reconstitution into a nucleosome-like particle. Further we show that the pyrene excimer fluorescence which results from the close proximity of two pyrenes in the same nucleosome depends on the ionic strength of the solution and agrees well with other studies on the conformation of the nucleosome in its native form (Ausio et al., 1984; Uberbacher et al., 1983) and when containing dansyl-labeled histone H4 (Lewis, 1979; Chung & Lewis, 1985).

Materials and Methods

Preparation of Core Nucleosomes and Nucleosomal DNA. Chromatin (100 A_{260} units/mL) was isolated from fresh chicken erythrocytes by the method of Olins et al. (1975) and subsequently digested by micrococcal nuclease (100 units/mL) for 1 h at 37 °C in 1 mM $CaCl_2$, 10 mM tris(hydroxymethyl)aminomethane (Tris), and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4, following a modification of the Lutter (1978) procedure as follows. After digestion and centrifugation, soluble chromatin was extracted from the pellet with 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM PMSF, pH 7.4. Histones H1 and H5 were then removed by column chromatography on Bio-Gel P100 (0.65 M NaCl and 10 mM Tris, pH 7.4). The H1/H5 stripped chromatin (25 A_{260} units/mL) was redigested for 40 min with micrococcal nuclease (100 units/mL) in 0.1 mM $CaCl_2$, 10 mM Tris, and 1 mM PMSF, pH 7.4. The reaction was terminated by the addition of EDTA to 10 mM and the digest mixture fractionated by gel filtration on Sephacryl S-300 eluted with 10 mM Tris–cacodylate and 0.7 mM EDTA, pH 7.4.

DNA was isolated from the core nucleosomes by chromatography on hydroxylapatite (Simon & Felsenfeld, 1979) as follows: 50 A_{260} units of core nucleosomes were brought to 2.5 M NaCl and applied to a hydroxylapatite column (1.5 × 4.0 cm). The column was washed with 2.5 M NaCl and 0.1 M phosphate, pH 6.0, to remove unbound histones, and then the DNA was eluted with 0.5 M phosphate, pH 6.0. The DNA was dialyzed into 10 mM EDTA, pH 7.4, and stored at 4 °C. Recoveries were greater than 80%, and all samples used had greater than 30% hyperchromicity as determined from thermal denaturation measurements. This procedure gave homogeneous, nick- and protein-free 146 ± 5 base pair (bp) DNA fragments when sized against *Hae*III restriction fragments of pBR322 DNA.

Preparation of Histones. Electrophoretically homogeneous histones H3 and H4 and an equimolar mixture of H2A and H2B were isolated from chicken erythrocyte acid-extracted histones depleted of H1/H5 by gel filtration on Bio-Gel P10 (Van der Westhuyzen et al., 1974) as follows. Frozen erythrocyte nuclei (15 g) were sheared in a Waring blender in 100 mL of 1 mM EDTA, 1 mM PMSF, 1% thiodiglycol, and 0.4 N HCl. The suspension was centrifuged and the pellet reextracted. The histones from the pooled supernatants were precipitated with 7% perchloric acid (PCA), pelleted, and

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washed several times with 7% PCA. The final pellet was washed once with acetone (0.1% HCl) and twice with dry acetone and then vacuum-dried. The yield was about 800 mg of protein. The histones were fractionated by gel filtration on Bio-Gel P10. Appropriate fractions were pooled, concentrated by ultrafiltration (Amicon PM10 filter), lyophilized, and finally redissolved in 20 mM HCl. Protein concentrations were determined spectroscopically by using published extinction coefficients (D'Anna & Isenberg, 1974), and their purity was determined on 15% acid–urea or 15% sodium dodecyl sulfate (SDS)–polyacrylamide slab gels (Panyim & Chalkely, 1969; Thomas & Kornberg, 1975).

Pyrene Labeling of Histone H4. In a typical labeling experiment, 2 mg of histone H4 was dissolved in 100 μL of water and the pH adjusted to 3.5 with glacial acetic acid. 1-*N*-Pyrenyliodoacetamide (2 mg), dissolved in 500 μL of dimethylformamide, was slowly added to the histone solution and the reaction allowed to proceed for 48 h in the dark at 37 °C. The pH was checked periodically and never exceeded 3.7. This ensured the specificity of the iodoacetamide for methionine and minimized acid hydrolysis of the histone. The labeled histone was precipitated by the addition of 6 volumes of acetone, and unreacted label was removed by repeatedly washing the precipitate with 1-mL aliquots of dimethylformamide/0.5% HCl. The amount of bound label was determined by the absorbance at 343 nm in 4 M guanidine hydrochloride (Gdn-HCl), 1 M NaCl, 5 mM Tris, and 1 mM EDTA, pH 7.5, using a molar extinction coefficient of 34 000 M^{-1} cm^{-1} (Betcher-Lange & Lehrer, 1978). The protein concentrations were assayed according to Lowry et al. (1951).

Histone H4 was also modified specifically at lysines with the *N*-hydroxysulfosuccinimidyl monoester of dipyrenyl-L-cystine (DPC-NHSS). The reagent was prepared in situ by reacting *N*,*N*′-dipyrenyl-L-cystine, *N*-hydroxysuccinimide sulfonate, and dicyclohexylcarbodiimide (1:1:1.1 molar ratios) in dimethylformamide at room temperature for 18 h. The *N*,*N*′-dipyrenyl-L-cystine was prepared by the reaction of 1-pyrenesulfonyl chloride with L-cystine in tetrahydrofuran/water/triethylamine (8:4:1). A 10-fold molar excess of DPC-NHSS was used to label histone H4 in 0.5 M borate, pH 8, with less than one adduct per histone molecule. The reaction was allowed to proceed in the dark for 60 min at room temperature. The labeled H4 was recovered by precipitation with 7% PCA and washed exhaustively with acetone (0.1% HCl). After a final acetone wash, the pellet was vacuum-dried, redissolved in 10 mM HCl, and further enriched for labeled protein by chromatography on Bio-Gel P6 (1.0 × 6.0 cm) equilibrated with 10 mM HCl. The pyrenyl-labeled histone elutes with the total volume of the column.

Aspartate Cleavage of Pyrene-Labeled H4. Five hundred micrograms of protein was dissolved in 250 μL of 0.25 M acetic acid, sealed in a glass tube, and incubated at 100 °C for 24 h. The peptides were separated by gel filtration on Sephadex G-50 (10 mM HCl), and the absorbance at 220 nm as well as the relative fluorescence at 400 nm (excited at 340 nm) was determined. Peptide purity was established by electrophoresis on 30% acid–urea slab gels stained with amido black (Lewis et al., 1975). The purified peptides were identified by amino acid analyses.

Nucleosome Reconstitution. Reconstitution experiments using purified DNA, histones, and labeled H4 were carried out as described elsewhere (Chung & Lewis, 1985). Our previous studies with a dansyl group bound to Met-84 of H4 show that this procedure results in labeled nucleosomes that are virtually indistinguishable from native particles by a variety of criteria (Lewis, 1979; Lewis & Chiu, 1980; Chung & Lewis, 1985). The reconstituted nucleosomes, after purification on 5–20% sucrose gradients, were routinely checked for their homogeneity, mobility on 5% TCE (10 mM Tris–cacodylate and 0.7 mM EDTA, pH 7.4) polyacrylamide gels (Maniatis et al., 1975), histone integrity, and stoichiometry. Furthermore, these particles were examined by thermal denaturation (Lee et al., 1982) and DNase I digestion (Lutter, 1978) to determine whether the pyrene groups detectably perturb the overall structure.

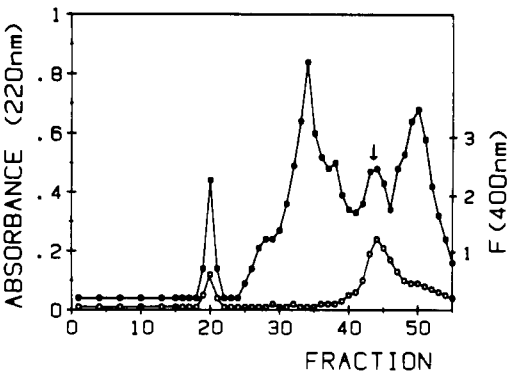

FIGURE 1: Gel filtration of aspartic acid cleaved histone H4 labeled at Met-84 with 1-*N*-pyrenyliodoacetamide on Sephadex G-50. (●) Absorbance at 230 nm; (○) relative fluorescence at 400 nm. Excitation at 340 nm. The arrow indicates H4 peptide 69–84.

Fluorescence Measurements. Spectra of samples in 3-mm rectangular cuvettes were obtained from an Aminco SPF 500 spectrofluorometer. Emission wavelengths and buffer conditions are given in the figure legends. Nucleosome concentrations were in the range from 1×10^{-7} to 5×10^{-7} M, unless otherwise specified. Spectra for the modified histones in free solution were obtained at a concentration of 50 μg/mL protein. The excitation wavelength and slit widths were 340 and ±5 nm, respectively. Precise temperature control was maintained, and time was allowed for thermal equilibration between measurements.

Chemical Cross-Linking of Pyrene-Labeled Nucleosomes. The histone octamer was cross-linked in situ with dimethyl 3,3′-dithiobis(propionimidate) (DMDP; Rosen et al., 1983). Core nucleosomes at 10^{-7} M were adjusted to 40 mM DMDP and 10 mM borate, pH 9.5. The mixture was incubated at 20 °C for 90 min and the pH lowered to 7.5 with glacial acetic acid. Following exhaustive dialysis against 10 mM TCE, the cross-linked nucleosomes were concentrated by ultrafiltration using an Amicon PM10 filter. SDS–polyacrylamide gel electrophoresis (PAGE) confirmed that the histones had been cross-linked into a single species with the mobility expected for an octameric complex. The reaction conditions used were similar to those described by Thomas and Kornberg (1975) for cross-linking of the histone octamer.

Results and Discussion

Pyrene-Labeled Histone H4. We have shown previously that histone H4 can be specifically modified at low pH at the single methionine at position 84 with dansylated iodoacetamide (Lewis, 1979). In this study, a pyrene group has been similarly attached to this site. The extent of labeling is about 70% at 48 h of reaction and increases to 95% at 96 h. To confirm the specificity of methionine labeling, the pyrene-labeled H4 was completely hydrolyzed at its three aspartic acid residues, and the four peptides were isolated by gel filtration on Sephadex G-50 as shown in Figure 1. More than 70% of the pyrene fluorescence elutes with the peptide peak which was

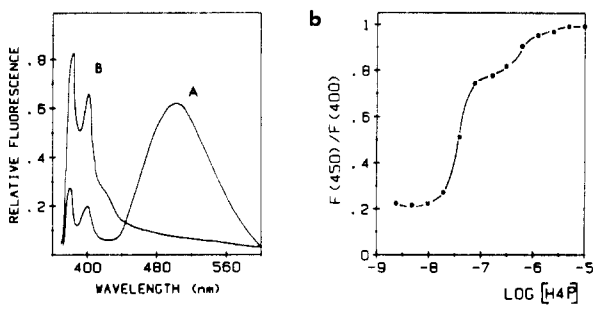

FIGURE 2: Fluorescence emission and association properties of pyrene-labeled histone H4. (a) Corrected emission spectra of H4 labeled with DPC-NHSS (A) and 1-*N*-pyrenyliodoacetamide (B) in 10 mM Tris, pH 7.4. (b) Dependence of excimer (450 nm) to monomer (400 nm) fluorescence intensity ratio on monopyrenyl-Met-labeled histone H4 concentration. Excitation at 340 nm.

previously identified as peptide 69–84 by its size and amino acid composition (Lewis et al., 1975). The trailing pyrene fluorescence is probably due to hydrophobic interaction of the 69–84 peptide with the column matrix, a phenomenon which occurs with other large aromatic groups (P. N. Lewis and D. G. Chung, unpublished results). No detectable labeling occurs at lysine, as peptide 1–23, which contains four modifiable lysine residues and elutes at fraction 36, has very little pyrene fluorescence.

Histone H4 was also labeled with a second reagent, DPC-NHSS, which reacts specifically with lysine residues. The adduct acylates lysine side chains and introduces the DPC moiety which contains two pyrene groups separated by a disulfide bridge. The distance between the pyrene groups can vary from contact to 12 Å depending on the values of the dihedral angles. There are 11 lysine residues in histone H4, and they appear, from the aspartic acid peptides, to be labeled at random by the acylating reagent (data not shown).

Fluorescence Properties of Pyrene-Labeled Histone H4. The emission spectra of the Met-84-labeled monopyrenyl H4 and the Lys-labeled dipyrenyl H4 are shown in Figure 2a. In each sample, there is less than one adduct per H4 molecule. The monopyrenyl H4, at low concentration, displays the usual pyrene spectrum (Birks, 1970) of two emission maxima at 380 and 400 nm. The dipyrenyl H4 displays an entirely different spectrum with a broad emission band centered aroud 500 nm. This emission is due to the formation of an excimer (excited dimer) caused by the close proximity of two pyrene groups (Birks, 1970; Zama et al., 1977). The two pyrene groups in the covalently bound DPC moiety are able to approach each other to form the excimer complex. For the DPC label, the excimer occurs irrespective of the H4 concentration as the interaction is intramolecular.

The monopyrenyl-Met-84-modified H4, we found, has a strong tendency to self-associate in solutions where its concentration exceeds 10^{-7} M as indicated by turbidity and the presence of concentration-dependent excimer fluorescence. The ratio of excimer to monomer fluorescence as a function of the monopyrenyl H4 concentration is shown in Figure 2b. The extent of Met-84 labeling in this particular sample was 95%. There is a broad transition with a midpoint around 3×10^{-7} M which probably reflects the dissociation of aggregated H4. As the concentration is lowered even further, all the excimer fluorescence is lost during a highly cooperative transition centered at 4×10^{-8} M. Clearly, the affinity of pyrene-labeled proteins for each other must be taken into consideration when working at concentrations greater than 10^{-7} M in dye. This is in agreement with the results of Prior et al. (1980), who have also found nonrandom association of pyrene-labeled histone H3 at similar concentrations. However, at concentrations of dye less than 10^{-7} M, pyrene-labeled H4 seems to incorporate randomly and more efficiently into core nucleosomes during reconstitution. The resulting fluorescence emission spectrum exhibits a mixture of monomer and excimer forms of pyrene (see Figure 10). Since pyrene fluorescence emission is detectable in the nanomolar range, it is quite feasible to obtain useful data about macromolecular interactions without the interpretations being complicated by the hydrophobic affinities of the fluors for each other.

Preparation of Pyrene-Labeled Nucleosomes. As we have shown previously, histone H4 modified at Met-84 with a dansyl group together with unlabeled H2A, H2B, and H3 and core length DNA is efficiently incorporated into nucleosome-like particles (Lewis, 1979). In contrast, pyrene-labeled H4 is only incorporated into a nucleosome-like particle under certain conditions. This is probably because of the above-mentioned aggregation properties of pyrenyl H4. If highly monolabeled (>80%) H4 is used during the reconstitution procedure, virtually all of the pyrene fluorescence as well as histone H4 is lost during the last centrifugation step prior to fractionation on a sucrose gradient. The elution profile of the sucrose gradient in this case shows mostly free DNA and very little reconstituted nucleosome. Clearly, the pyrene-labeled H4 aggregated during the reconstitution procedure since the concentration of dye exceeded 10^{-7} M. Thus, the labeled H4 was pelleted during the above centrifugation step. Since it was desirable to carry out reconstitutions at a high concentration of histone, several different approaches were tried to circumvent this problem. The first was to reconstitute nucleosomes using poly(glutamic acid) as an assembly factor (Retief et al., 1984). The second was to include pyrene sulfonate, a water-soluble analogue of the pyrene reagent, in the reconstitution mixture. This analogue is unable to react with methionine but might have acted as a carrier to prevent aggregation by competing with pyrenyl H4. Neither of these methods was successful. However, pyrene-labeled nucleosomes were successfully reconstituted at lower concentrations of labeled histone H4.

A series of assembly experiments were carried out under the same conditions as above. However, the monopyrenyl-labeled H4 was now mixed with increasing amounts of unlabeled H4. We found that if the ratio of monolabeled H4 to unlabeled H4 was greater than 1, then very little nucleosome was obtained. For ratios below 0.7, properly reconstituted nucleosomes were observed in the sedimentation profile. The yield in terms of the amount of DNA recovered as an 11S nucleosome was 70% of the input, and most of the fluorescence comigrated with the nucleosome peak on the sucrose gradient (Figure 3a, curve C). The absorbance at 260 nm of the gradient elution profile (Figure 3a, curve B) is the same as profiles obtained from reconstitution mixtures containing unmodified H4 and H4 modified with other fluorescent groups (Lewis, 1979; Chung & Lewis, 1985). For comparison, curve A in Figure 3a is the gradient elution profile (absorbance at 260 nm) resulting from an equimolar mixture of free DNA and native core nucleosomes. These reconstituted nucleosomes recovered by using this procedure are very similar to control reconstituted (containing unmodified H4) nucleosomes as well as native nucleosomes as judged by a variety of criteria such as thermal denaturation and DNase I digestion (data not shown). The *s* value of the pyrene-containing nucleosome is the same as that of native core nucleosomes (11 S). These monopyrenyl H4-labeled nucleosomes are also indistinguishable from native nucleosomes, as judged by their electropho-

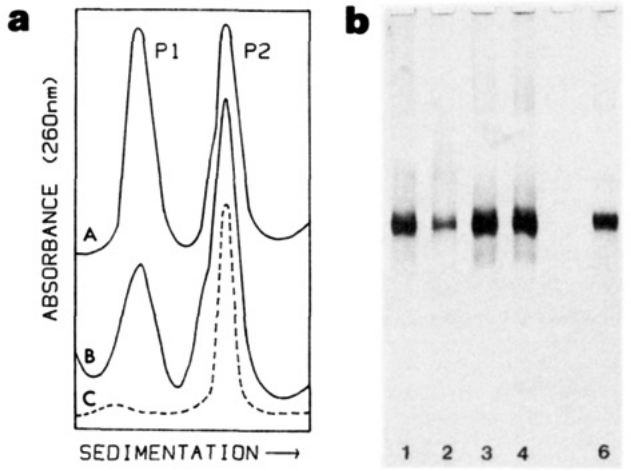

FIGURE 3: Sucrose gradient sedimentation profile and 5% TCE particle gel analysis of pyrene-labeled nucleosomes. (a) Sucrose gradient sedimentation. Free DNA and core nucleosomes sediment at positions P1 and P2, respectively. (A) is the A_{260} profile resulting from a mixture of core length DNA and native core nucleosome. (B) is the profile resulting from a reconsitution mixture containing monopyrenyl-Met-84-labeled H4. (C) shows the relative fluorescence emission at 400 nm of the reconstitution mixture. The excitation was at 340 nm. (b) 5% TCE particle gel mobility of pyrene-labeled and native core nucleosomes. Lane 1, nucleosomes containing 10% monopyrenyl-Met-84-labeled H4. Lane 3, nucleosomes containing 40% monopyrenyl-Met-84-labeled H4. Lanes 2 and 4 have the same composition as the samples in lanes 1 and 3, respectively, but the Cys-110 of the two H3 molecules is in the intermolecularly oxidized form. Lane 6, native core nucleosome.

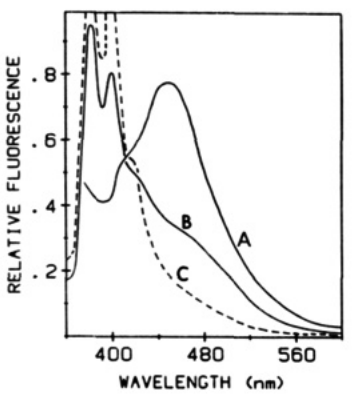

FIGURE 4: Fluorescence emission spectra of core nucleosomes containing dipyrenyllysine-labeled H4. Curve A is the spectrum in 10 mM Tris, pH 7.4; curve B in 10 mM Tris and 10 mM DTT, pH 7.4; curve C in 10 mM Tris, 10 mM DTT, and 0.2% SDS, pH 7.4. The excitation was at 340 nm in all cases.

retic mobility on 5% TCE particle gels as shown in Figure 3b, and also contain a full complement of undegraded histones (data not shown).

Effect of Denaturants on Pyrene Excimer Fluorescence. The fluorescence emission spectra of the dipyrenyl-labeled H4-containing nucleosomes (randomly labeled at lysine residues) are shown in Figure 4 for a variety of conditions. As expected, we observe only excimer fluorescence (Figure 4, curve A), but the emission spectrum has been shifted relative to that seen for the labeled H4 in Figure 2a. This blue shift is most likely due to incorporation of the dye into the hydrophobic interior (Prior et al., 1980), and our data therefore indicate that most of the labeled sites of histone H4 are inaccessible to the solvent. Upon addition of dithiothreitol (Figure 4, curve B), most of the pyrene excimer fluorescence collapses to that of the monomer as half of the pyrenes are cleaved from the DPC to yield a monopyrenyl group. The

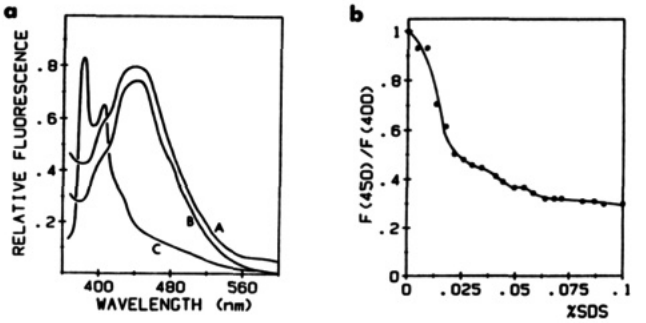

FIGURE 5: SDS unfolding of core nucleosomes containing monopyrenyl-Met-labeled H4 as monitored by fluorescence emission. (a) Curve A is the emission spectrum of dipyrenyl H4 nucleosomes. Curve B is the emission spectrum of monopyrenyl H4 nucleosomes in 10 mM Tris, pH 7.4. Curve C is due to same sample as in curve B but in 10 mM Tris and 0.2% SDS, pH 7.4. (b) SDS concentration dependence of the excimer to monomer intensity ratio of monopyrenyl H4 nucleosomes. Excitation at 340 nm.

small residual excimer fluorescence is most likely due to cleaved pyrenes interacting hydrophobically with their counterparts (still covalently attached to the histone) or less likely (see below) to the close proximity of two lysine residues that have both been modified with the dipyrenyl-L-cystine compound. The exposure of these nucleosomes to 0.2% SDS (Figure 4, curve C) results in the complete loss of excimer fluorescence by breaking the hydrophobic interactions mentioned previously. If the remaining excimer fluorescence were due to pyrene molecules situated on adjacent lysine residues, then SDS should not completely abolish pyrene excimer fluorescence.

The emission spectrum of nucleosomes containing H4 labeled at Met-84 with 1-*N*-pyrenyliodoacetamide is shown in Figure 5a, curve B. In this particular sample, only excimer fluorescence is observed. The spectrum is essentially the same as that obtained for H4–DPC nucleosomes (Figure 5a, curve A). In some samples, a mixture of monomer and excimer fluorescence emission is observed. In either case, the two pyrene molecules exhibiting excimer formation are not chemically linked to each other. Thus, the excimer fluorescence must arise from the close proximity of the two Met-84 sites on the H4 molecules to which the pyrenes are attached. If this is the case, then unfolding of the nucleosomal structure by denaturants such as SDS and guanidine should result in the loss of excimer fluorescence. This is shown in Figure 5a, curve C, where the monopyrenyl H4 nucleosome has been dissociated in 0.5% SDS. At this concentration of SDS, the histones are no longer in contact with the DNA and have also dissociated from each other. As expected, the fluorescence spectrum changes to that of the monomer form of pyrene. An SDS titration was carried out, and the results are shown in Figure 5b. There is an initial steep drop in the ratio of excimer to monomer emission centered at 0.01% SDS followed by a much broader transition between 0.025% and 0.075% SDS. This second transition is very similar to one observed with nucleosomes containing dansylated H4 (Chung & Lewis, 1985). During SDS titration of the dansylated nucleosome, there is an initial increase in the fluorescence as the solvent is made less polar. This is followed by a broad but cooperative transition, centered around 0.05% SDS, where the fluorescence emission decreases as the Met-84 site of H4 becomes progressively more exposed to solvent. From this, we suggest that the first pyrene transition results from the local unfolding of the H4–H4 contact region, while the broad second transition is due to the dissociation of H4 from the histone octamer complex.

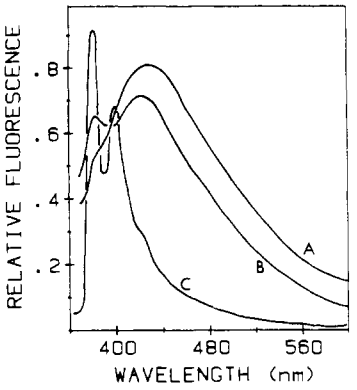

FIGURE 6: Fluorescence emission spectra of monopyrenyl-Met-labeled H4 nucleosomes after chemical cross-linking of the histone octamer. Curve A, 10 mM Tris, pH 7.4; curve B, 10 mM Tris and 0.2% SDS, pH 7.4; curve C, 10 mM Tris, 0.5% SDS, and 10 mM DTT, pH 8. Excitation at 340 nm.

Chemical Cross-Linking of the Histone Octamer. Pyrene groups have a considerable hydrophobic affinity for each other, and thus the observed excimer band might be due to unbound pyrene molecules, which may remain even after dialysis and sucrose gradient centrifugation, rather than the interaction of two pyrene groups bound to the two Met-84 side chains. To distinguish between these possibilities, the histone octamer was cross-linked in situ. The cross-linker was a dimethyl suberimidate derivative which is cleavable by thiol reagents by virtue of a disulfide bridge in the center of the molecule. It was verified by SDS–polyacrylamide gel electrophoresis that the cross-linking procedure used resulted in the formation of a cross-linked histone octamer. If the excimer is not due to pyrene molecules bound covalently to the Met-84 sites of H4, then placing the cross-linked nucleosomes in 0.5% SDS should result in the loss of the excimer fluorescence because any noncovalently bound pyrene groups can dissociate from each other under the influence of the denaturant. The emission spectrum of monopyrenyl-Met-84 H4-labeled nucleosomes after chemical cross-linking is shown in Figure 6, curve A. Almost all the excimer fluorescence has been retained, but the peak emission has been shifted by about 10 nm to shorter wavelengths. This may be due to changes in polarity around Met-84 of H4 caused by the cross-linking reaction. The emission spectrum of the cross-linked nucleosome in 0.5% SDS is shown in Figure 6, curve B. This concentration of the denaturant is sufficient to disrupt the structure of an uncross-linked nucleosome and cause the loss of all excimer fluorescence (Figure 5, curve C). However, the emission spectrum is only minimally affected by this treatment, thus proving that the observed excimer fluorescence is due to the close proximity of two pyrene groups covalently bound to the two H4 Met-84 sites within the nucleosome. When the SDS-containing cross-linked nucleosome sample is treated with 10 mM 2-mercaptoethanol at pH 8.5 to disrupt the cross-links, curve C in Figure 6 is obtained. All of the excimer fluorescence has collapsed into a spectrum characteristic of pyrene monomer emission. Since the thiol reagent cleaves the disulfide bridges of the cross-linker, the histones are no longer covalently attached to each other and are thus dissociated by SDS. The two Met-84 sites of the H4 molecules move apart which results in the loss of the pyrene excimer fluorescence.

Structure of the Pyrene Excimer. The results presented above demonstrate the close proximity of the two labeled H4 molecules, or at least their C-terminal regions, within the nucleosome. Structural analyses of crystalline pyrene show that the molecules are arranged in offset but overlapping pairs

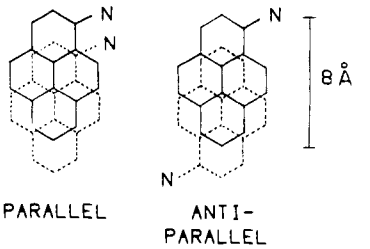

FIGURE 7: Structure of the pyrene excimer. Both configurations are front to back arrangements as shown in Zama et al. (1977). The bar denotes 8 Å.

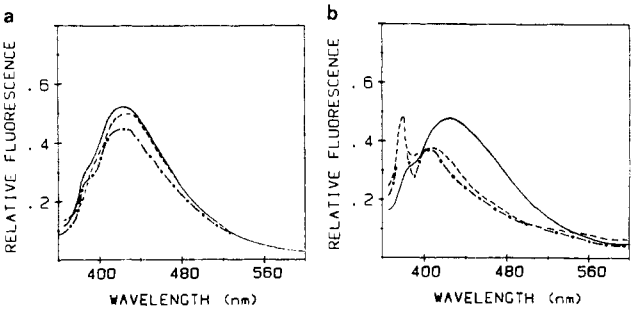

FIGURE 8: Effect of ionic strength on the fluorescence emission of monopyrenyl-Met-labeled H4 nucleosomes. (a) Emission spectra are shown for samples at 0.1 (—), 1.0 (●–●), and 10 (---) mM NaCl. (b) Emission spectra are shown for samples at 100 (—), 350 (---), and 600 (●–●) mM NaCl. The excitation was at 340 nm, and the various salt solutions also contained 0.1 mM Tris, pH 7.4.

(Birks, 1970; Zama et al., 1977). Figure 7 shows projections of two possible excimer configurations. In the unexcited state, the equilibrium interplanar distance is 3.53 Å, and the two molecules are offset by 1.75 Å relative to each other. Thermodynamic studies indicate that these stringent geometric constraints must be met before excimer fluorescence can occur (Birks, 1970; Zama et al., 1977). Thus, if we assume that these constraints apply to the observed excimer of pyrene-labeled nucleosomes, we can estimate the distance separating Met-84 sites on the two histone H4 molecules. Zama et al. (1977) have identified at least eight possible configurations. The two arrangements shown in Figure 7 represent the extremes in distance between the two pyrene molecules. For excimer formation, the distance between the methionine sulfur atoms of the two histone H4 molecules would therefore range from 5 to 14 Å.

Effect of Low Ionic Strength on Pyrene Excimer Fluorescence. Many studies have been made of the low ionic strength behavior of the nucleosome. There is still some controversy regarding the unfolding of nucleosomes below 1 mM ionic strength. Cantor and co-workers conclude that the histone octamer becomes very flexible at low ionic strength on the basis of the emission behavior of fluorescent dyes attached to Cys-110 of histone H3 (Dieterich et al., 1977, 1979). Uberbacher et al. (1983), on the other hand, observed only small conformational changes in the internal core structure when investigating the same transition using neutron scattering.

We have recently reported a fluorescence emission study of nucleosomes containing histone H4 that have been modified with a dansyl derivative at Met-84 (Chung & Lewis, 1985). It was found that the histone octamer does not undergo major structural changes when transferred into a low ionic strength buffer, although several discrete conformational transitions in the range from 0.1 to 600 mM NaCl were nevertheless observed. By examining the effect of ionic strength in this same range on the excimer emission of monopyrenyl-Met H4-labeled nucleosomes, one can determine whether the

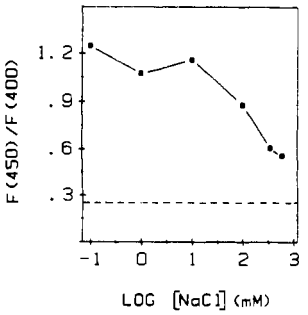

FIGURE 9: Ionic strength dependence of the excimer to monomer fluorescence intensity ratio of monopyrenyl-Met-labeled H4 nucleosomes. The dashed line represents the limiting value of this ratio when there is no observable excimer.

changes observed around one H4 locus are accompanied by the relative movement of one H4 molecule with respect to the other as reflected in the loss of excimer fluorescence.

The emission spectra of monopyrenyl-Met H4-labeled nucleosomes at 0.1, 1, and 10 mM NaCl are shown in Figure 8a. The nucleosomes in the sample used for this particular study exhibited mostly excimer fluorescence (both H4 molecules labeled per nucleosome) but showed some monomeric pyrene (only one H4 molecule labeled per nucleosome) as well. As the ionic strength is increased, the pyrene excimer (450 nm) to monomer (400 nm) ratio of emission intensities decreases from 1.25 at 0.1 mM NaCl to 1.08 at 1 mM NaCl and then increases again to 1.16 at 10 mM NaCl. The value of this ratio for a spectrum consisting only of monomer fluorescence in pyrenyl H4 nucleosomes is 0.25. The approximately 10% decrease in this ratio from 0.1 to 1 mM NaCl closely parallels changes found previously for an environment-sensitive dansyl group attached to this same site (Chung & Lewis, 1985). This we feel provides evidence that the two interacting pyrene groups do not perturb the structure of the nucleosome any more than a single dansyl group does. The simplest explanation for these data is that the histone octamer, at least around Met-84 of H4, is maximally folded at both 0.1 and 10 mM NaCl but becomes somewhat perturbed at 1 mM NaCl. Even so, because a large proportion (>85%) of the excimer is retained, we conclude that the histone octamer remains intact at low ionic strength. Large conformational changes involving the dissociation of the H4–H4 contact region are unlikely in view of the geometric constraints required to maintain two pyrene groups in an excimer configuration. To support this interpretation, we have measured the circular dichroism spectra of mononucleosomes at low ionic strengths (data not shown) and find that the α-helical content remains essentially constant for the entire range of salt concentrations.

Effect of Moderate Ionic Strength on Pyrene Excimer Fluorescence. Exposure of the monopyrenyl-Met-84 H4-labeled nucleosomes to ionic strengths greater than 10 mM results in a dramatic loss of excimer fluorescence, as shown in Figure 8b. The ratio of excimer to monomer fluorescence intensity as a function of ionic strength is shown in Figure 9. By 100 mM NaCl, the excimer to monomer ratio has decreased by 30%. At 600 mM salt, a roughly 60% drop in excimer fluorescence is observed. The dashed line in Figure 9 indicates the limiting value of the excimer to monomer fluorescence intensity ratio when there is no more observable excimer. The presence of residual excimer fluorescence (Figures 8b and 9) at 600 mM NaCl suggests that the H4–H4 contact, while largely disrupted, is still in dynamic equilibrium with the folded state. Taken together with the results of Ausio et al. (1984), who have shown that the histone octamer remains relatively intact and bound to the DNA up to 0.6 M monovalent salt, we suggest that the 0.6 M NaCl structure around Met-84 on H4 is not dramatically different from the 10 mM structure.

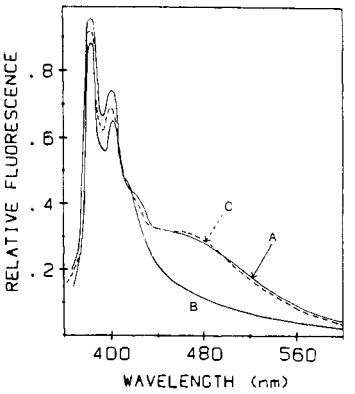

FIGURE 10: Reversibility of ionic strength induced structural changes in monopyrenyl-Met-84 H4-labeled nucleosomes. (A) is the emission spectrum in 10 mM Tris, pH 7.4. (B) is the emission spectrum in 10 mM Tris and 2 M NaCl, pH 7.4. (C) is the emission spectrum of sample B after dialysis into 10 mM Tris, pH 7.4. Excitation at 340 nm in all cases.

We have also tested the reversibility of the high-salt-induced structural changes in the histone octamer. The sample used for this particular study contained a relatively large proportion of monomeric pyrene, but excimer fluorescence is nevertheless evident (Figure 10, curve A). Increasing the ionic strength to a final concentration of 2 M NaCl resulted in the complete loss of the excimer fluorescence (curve B). The emission spectrum of monopyrenyl-Met-84 nucleosomes after exhaustive dialysis into 10 mM Tris is shown in curve C. All of the excimer fluorescence has been regained after the loss at high ionic strength. This indicates complete reversibility of the salt-induced structural transitions, at least when monitored at the Met-84 locus of histone H4.

At 2 M monovalent salt, the histone core has dissociated from the DNA and exists primarily as an octameric species. Since the 0.6 M NaCl spectrum shows some residual excimer fluorescence, whereas the 2 M NaCl spectrum does not, the octamer may be less compact at the higher salt concentration. This is in agreement with the results of Burlingame et al. (1985), who have recently crystallized the histone octamer from 2 M NaCl. The dimensions obtained for the octamer are about twice as large as the values obtained from other X-ray structures of the core nucleosome (Richmond et al., 1984; Uberbacher & Bunick, 1985).

Relationship to Studies with H3-Labeled Nucleosomes. Modification of the H4 Met-84 loci in the core nucleosome by bulky fluorescent reporter groups does not appear to perturb its structure. All of the physical properties of reconstituted nucleosomes containing pyrene-modified H4 are the same as those of native core particles. In the ionic strength range from 0.1 to 10 mM NaCl, the sulfur atoms of H4 Met-84 are within 5–14 Å of each other and undergo only minor changes around 1 mM monovalent salt. We find no evidence in this ionic strength range for a flexible and expanded structure for the histone octamer as indicated by studies using histone H3 labeled with similar fluors at Cys-110 (Cantor et al., 1981). Beyond 10 mM monovalent salt, there is a progressive increase in the exposure and mobility of other fluors bound to H4 Met-84 (Chung & Lewis, 1985). However, even by 0.6 M monovalent salt, these changes are much less than those for free histones, urea-unfolded nucleosomes, or H3 Cys-110-labeled nucleosomes (Dieterich et al., 1979). We conclude that while there are detectable changes in the histone octamer at

the H4–H4 interface, they are inconsistent with a complete unfolding of the octamer as proposed from the labeled histone H3 studies (Cantor et al., 1981) and speculate that the differences between our results and those found for the H3-labeled nucleosomes are due to the apparent destabilization of nucleosomes containing H3 cysteine modified with reporter groups (Lewis & Chiu, 1980; Wingender et al., 1981; Ausio et al., 1984).

Registry No. DPC-NHSS, 100839-37-0; *N,N'*-dipyrenyl-L-cystine, 100839-38-1; *N*-hydroxysuccinimide sulfonate, 100839-39-2; 1-pyrenesulfonyl chloride, 61494-52-8; L-cystine, 56-89-3; 1-*N*-pyrenyliodoacetamide, 76936-87-3.

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