Singlet–Singlet Energy Transfer Studies of the Internal Organization of Nucleosomes†

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ABSTRACT: We report the measurement of two specific protein to DNA distances in several conformational states of core nucleosomes by singlet–singlet energy transfer. A distance of 50–53 Å separates each DNA terminus from cysteine-110 of chicken erythrocyte histone H3 in the native nucleosome. This cysteine residue must therefore be located very near the center of the nucleosome. The H3–DNA distance remained nearly constant in several unfolded forms of the core particles, as found in very low salt, in 0.6 M NaCl, and in high urea. Furthermore, it was shown that each DNA end lies within 32 Å of cysteine-73 of *Arbacia lixula* sperm histone H4 in both the compact and the low-salt unfolded forms of the nucleosome. Because of the invariance of the two measured distances in the various conformational states of the nucleosome, we conclude that the cysteine-containing C-terminal segments of histones H3 and H4 maintain a very strong and close association with the terminal positions of the 146 base pair nucleosomal DNA. This binding may provide the primary interactions necessary for the folding of DNA into nucleosomes and for protection of 146 base pair nucleosomes from further nuclease digestion.

The existence of a strong structure–function relationship in the eucaryotic nucleosome is implied by a remarkable conservation in the primary amino acid sequence of the histones (Delange & Smith, 1972). The increased nuclease sensitivity of the DNA in highly transcribed regions of the genome (Garel & Axel, 1976; Weintraub & Groudine, 1976) suggests that conformational changes of nucleosomes may occur during cellular transcription and replication. These observations have stimulated extensive examination of the internal organization of nucleosomes and the molecular changes responsible for their multiple conformational states (Zama et al., 1978; Gordon et al., 1978; Crothers et al., 1978; Dieterich et al., 1979; A. E. Dieterich, H. Eshaghpour, D. M. Crothers, and C. R. Cantor, unpublished experiments; Wu et al., 1979).

In this report we describe the preparation of fluorescent nucleosomal core particles, labeled specifically at the 3′ DNA ends and at two specific sites in the protein core. These preparations have enabled us to measure two specific protein–DNA distances by singlet–singlet energy transfer. These distances were monitored in several different conformational states of the nucleosome. The results reveal a very strong and stable interaction between the nucleosomal DNA termini and the labeled protein sites, positioned on the C-terminal segments of histones H3 and H4.

Experimental Procedures

Preparation of Fluorescent-Labeled Nucleosomes. (*1*) *Isolation of Nucleosomal Core Particles.* Trimmed core particles from chick erythrocytes (CE)[1] were prepared by the extensive digestion of H1,H5-depleted chromatin with micrococcal nuclease (Klevan & Crothers, 1977; Tatchell & Van Holde, 1977). Frozen concentrated chicken blood was a product of Pel-Freeze Biologicals. CE nuclei were prepared by the method of Hymer & Kuff (1964). Nuclei were lysed in 0.2 mM Na_2EDTA, pH 8.0, by gentle stirring at 4 °C. The resulting chromatin was pelleted by low-speed centrifugation and resuspended in 10 mM Tris–cacodylate and 0.7 mM EDTA, pH 7.2, by using a Waring blender at 40 V for 2 min. Following the slow addition of a high-salt solution to 0.6 M, the very lysine-rich histones H1 and H5 were removed by the cation-exchange resin AG50W-X2 (Na^+) (Bolund & Johns, 1973). The resulting H1,H5-depleted chromatin was concentrated by ultrafiltration (Amicon) and subsequently dialyzed against 10 mM Tris–cacodylate and 0.7 mM EDTA, pH 7.2. Digestion with 100 units/mL micrococcal nuclease (Worthington Biochemicals) was carried out for 45–60 min at 37 °C following the addition of $CaCl_2$ to 1 mM. The optimum extent of digestion was determined by DNA gel electrophoresis of a pilot digestion experiment. The reaction was terminated by addition of excess EDTA and cooling on ice. The digest was fractionated by gel filtration on Bio-Gel A5M (Shaw et al., 1976); the resulting nucleosome peak was further purified by sedimentation in an isokinetic sucrose gradient (McCarty et al., 1974; Simpson & Whitlock, 1976). Centrifugation was for 16–20 h at 35000 rpm and 4 °C in a Beckman SW40 rotor.

(*2*) *Preparation of Nucleosomal DNA.* Trimmed nucleosomal particles were used to isolate large quantities of random-sequence DNA fragments with the relatively homogeneous length of 146 base pairs (bp) (Prunell et al., 1979). The nucleosomes were treated with 50 μg/mL protease (Sigma Chemical Co.) for 2 h at 37 °C in the presence of 1 M NaCl. The DNA was then purified by extraction with buffered phenol, followed by multiple extractions with ether, and subsequent precipitation with 2% sodium acetate and 2 volumes of 95% ethanol (Maniatis et al., 1975).

(*3*) *Preparation of Fluorescent-Labeled DNA.* The highly fluorescent dye 5-(iodoacetamido)fluorescein (IAF) (Molecular Probes, Plano, TX) was covalently attached to the 3′ termini of DNA fragments by a newly devised method (Eshaghpour & Crothers, 1979). Briefly, the basis for this pro-

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[1] Abbreviations used: CE, chick erythrocytes; bp, base pairs; IAF, 5-(iodoacetamido)fluorescein; IAEDANS, 5-[[(iodoacetamido)ethyl]-amino]naphthalene-1-sulfonic acid; TE, 10 mM Tris-HCl and 2 mM Na_2EDTA, pH 7.8; ME–IAF, β-mercaptoethanol–fluorescein.

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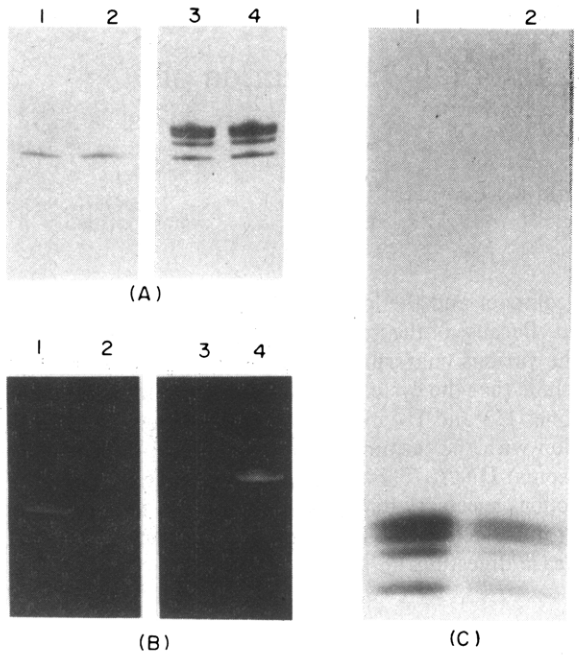

FIGURE 1: Histones were electrophoresed on discontinuous 15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Analysis of proteins prior to reconstitution: (A) Coomasie Brilliant Blue staining pattern and (B) IAEDANS emission of a gel containing (1) IAEDANS-labeled and (2) unlabeled *A. lixula* sperm histone H4 and (3) unlabeled and (4) IAEDANS-labeled chick erythrocyte core histones. (C) Histones present in (1) native CE nucleosomes and (2) reconstituted nucleosomes.

cedure is the specific addition of 4-thiouridine residues to the 3′-terminal hydroxyls of DNA using calf thymus terminal deoxynucleotidyltransferase, followed by treatment with pancreatic ribonuclease. The reactive thio group can then be modified by reaction in an aqueous solution with α-haloacetamido derivatives of dye molecules, in this instance IAF. When analyzed on polyacrylamide gels, the fluorescein emission exhibited the same electrophoretic mobility as the ethidium-stained DNA.

(*4*) *Preparation of Fluorescent-Labeled Histones.* Core histones were isolated from whole CE chromatin by acid extraction following published procedures (Panyim et al., 1971; Oliver et al., 1972). Chicken erythrocyte core histones contain only one cysteine residue in their primary sequence, amino acid 110 of histone H3 (Brandt & Von Holt, 1974). This amino acid was specifically labeled with the radioactive fluorescent label [^{3}H]-5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonic acid (IAEDANS) in 5 M urea and 2 M NaCl, pH 8.0 (Figure 1A,B) following the procedure previously described for the preparation of IAEDANS-labeled CE nucleosomes (Dieterich et al., 1978).

Fractionated histones H3 and H4 from the sperm of sea urchin (SU) *Arbacia lixula* were the generous gifts of Dr. A. Ruiz-Carrillo. The single cysteine residue at position 73 of SU histone H4 (Strickland et al., 1974) was also modified with [^{3}H]IAEDANS (Figure 1A,B).

A complex of histones H2A and H2B was isolated from CE core histones by the selective precipitation of the H3–H4 tetramer complex with ammonium sulfate at 70% saturation (Klevan et al., 1978). All histone concentrations were determined by absorbance at 275.5 nm. By use of molar extinction coefficients for each histone reported by D'Anna & Isenberg (1974), the values of 4.04×10^4, 4.04×10^3, 5.4×10^3, and 1.08×10^4 M^{-1} cm^{-1} were estimated for the histone octamer, SU H3, SU H4, and CE H2A–H2B complex, respectively.

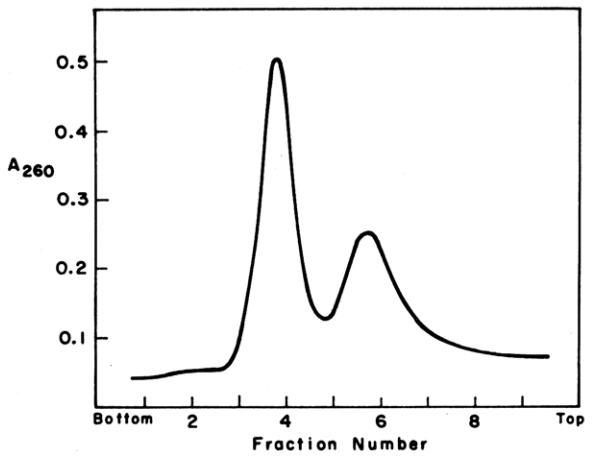

FIGURE 2: Isokinetic sucrose gradient fractionation of fluorescein-labeled nucleosomes. The major peak cosedimented with native CE core particles. Sedimentation was from right to left.

(*5*) *Reconstitution Procedure.* Fluorescent-labeled nucleosomal particles were reconstructed in vitro by the discontinuous salt gradient dialysis procedure (Axel et al., 1974; Oudet et al., 1975). The appropriately labeled DNA fragments and core histones were combined (1.0 mol of DNA per 1.25 mol of octamer) in 10 mM Tris-HCl and 2 mM Na_2EDTA, pH 7.8 (TE), in the presence of 5 M urea and 2 M NaCl. The final DNA concentration was 2–5 A_{260}/mL. Reconstitution was carried out by dialysis at 4 °C against TE and 5 M urea depending on the experiment and varying salt concentrations: first 2 M NaCl for 12–16 h and then 1.5 M NaCl for 3–5 h, 1.0 M NaCl for 3–5 h, 0.8 M NaCl for 8–12 h, and 0.6 M NaCl for 10–12 h, followed by the final dialysis against TE buffer for 16–24 h. All reconstitution experiments started with 5 M urea in the initial dialysis buffer. However, we experimented with reconstitutions both in the presence of 5 M urea through the 0.8 M NaCl dialysis step and in its absence following the initial 2 M NaCl step. The only difference that we have detected between these two procedures is the reduction of visible aggregates when urea is present throughout most of the steps of the gradient. Also, our yield of purified nucleosomes is usually better with this method of reconstitution.

Although we normally prefer to include 5–10 mM β-mercaptoethanol in our reconstitution buffers, no –SH reagents were used in these experiments. The sensitivity of the DNA–dye adduct to these reagents (Eshaghpour & Crothers, 1979) was the reason for their exclusion in the present series of experiments. We have previously shown that covalently attached fluorescein is removed from the DNA termini by extended treatment with β-mercaptoethanol (Eshaghpour & Crothers, 1979). In some of the reported experiments the nonfluorescent proteins were reacted with iodoacetamide prior to reconstitution to prevent the formation of disulfide bonds, making the use of reducing agents unnecessary.

The final reconstitution mixtures were fractionated on isokinetic sucrose gradients (Figure 2) as described above, from which, in addition to our 10.3S nucleosomal particles, we obtained variable amounts of a peak sedimenting at ~6.5–7 S and probably containing various partially reconstituted products.

Fluorescence Measurements. All fluorescence measurements were performed on a Schoeffel RRS-1000 spectrofluorometer interfaced to a Tektronix 31 programmable calculator. The sample temperature was maintained at ~25 °C with a Lauda K2R circulating water bath attached to the cell holder. All fluorescence experiments were carried out with

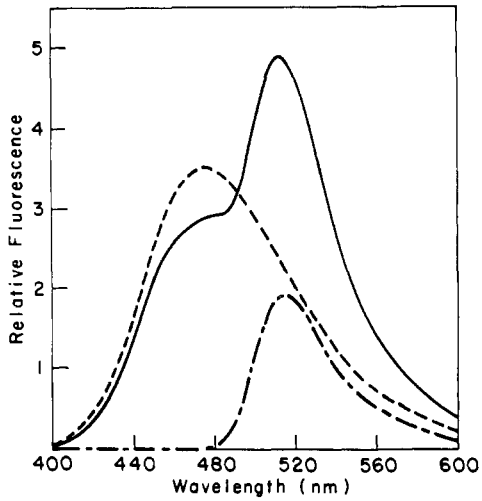

FIGURE 3: Emission spectra of fluorescently labeled nucleosomes in TE buffer (16 mM ionic strength). The various scans correspond to double-labeled nucleosomes DF containing both IAEDANS and fluorescein (–), IAEDANS-labeled nucleosomes D (- - -), and fluorescein-labeled nucleosomes F (–·–). The background signal from unlabeled nucleosomes was subtracted from all spectra. The excitation wavelength was 340 nm.

samples of 0.1–0.3 A_{260}/mL or (0.5–1.5) × 10^{-7} M nucleosomes. The samples were dialyzed extensively against TE buffer prior to fluorescence measurements. High-salt and urea experiments were carried out by the direct addition of 4 M NaCl or 10 M urea and TE stock solutions. Following a change in the sample medium, they were allowed to incubate for a minimum of 5–6 h at room temperature or overnight at 4 °C prior to the next set of fluorescence measurements. Samples for the low-salt experiments were prepared by initial dialysis against 1/50 × TE and 0.1 M KCl for 8–12 h, followed by two changes of 1/50 × TE buffer.

The quantum yield of IAEDANS in the various states of the nucleosome was measured relative to a value of 0.61 for H3–IAEDANS nucleosomes in TE buffer (Dieterich et al., 1979).

(*1*) *Energy Transfer Experiments.* One of the more facile means for detection of singlet–singlet energy transfer is by observing changes in the emission of the donor molecule [for a review of singlet–singlet energy transfer, see Fairclough & Cantor (1978)]. This requires a region of the donor emission spectrum with minimal contribution from the acceptor fluorescence. Excitation of our nucleosomal samples at 340 nm resulted in a strong emission of the donor IAEDANS between 400 and 600 nm, while the fluorescein emission becomes substantial only above 480 nm (Figure 3). The area of 440–480 nm in the emission spectra, therefore, presented an ideal region for the detection and quantitation of donor quenching.

Each energy transfer experiment required the simultaneous use of four nucleosome samples, designated by DF, D, F, and Blk, which differed only in their content of fluorescent probes. The double-labeled nucleosomes (DF) contained both the donor, IAEDANS-labeled histones, and the acceptor, fluorescein-labeled DNA; D nucleosomes contained only IAEDANS, F nucleosomes contained only fluorescein, and Blk nucleosomes lacked extrinsic probes and were used for fluorescence background. The concentration of the donor-containing nucleosomes was determined accurately by liquid scintillation counting after each experiment.

After the subtraction of background and correction for differences in concentration, the spectra were used for the direct measurement of the ratio of fluorescence intensities ($DF - F$)/D. This ratio was determined at 10-nm intervals between 440 and 480 nm. The average of these determinations gave an accurate measure for the ratio of the quantum yields of the donor IAEDANS in the presence (ϕ_{DF}) and absence (ϕ_D) of fluorescein within the nucleosome structure:

$$\frac{\phi_{DF}}{\phi_D} = \frac{DF - F}{D} \tag{1}$$

Once this ratio was obtained, the value for the observed efficiency of energy transfer (E_0) was determined through the relationship

$$E_0 = 1 - \frac{\phi_{DF}}{\phi_D} \tag{2}$$

(*2*) *Distance Determination.* In the compact form of the nucleosome, we were faced with a complex multiple donor–acceptor relationship, requiring special analysis of the experimental data. Specifically, we must allow for (a) incomplete labeling of the DNA and (b) the possibility of energy transfer from each donor to two acceptors.

The cysteine residues within the two copies of histone H3 in each nucleosome are very close in the compact form of the particle (Zama et al., 1978; Dieterich et al., 1979). This conclusion is supported by the success of Camerini-Otero & Felsenfeld (1977) in reconstructing nucleosomes with dimers of H3, linked through their thiol groups. Because it is very likely that the nucleosome has a C_2 axis of symmetry (see Results), the thiols must lie on the dyad axis, equidistant from both 3′ termini of the nucleosomal DNA in the compact form of the nucleosome.

The presence of two acceptor molecules equidistant from a donor is mathematically equivalent to one acceptor with twice the extinction coefficient (Gennis & Cantor, 1972). Thus, the effective extinction coefficient is

$$\epsilon_{490}^{\text{eff}} = 2\epsilon_{490}$$

The acceptor extinction coefficient enters Förster's theory of energy transfer through the 1/6 power of the critical distance (R_0). Therefore

$$(R_0^{\text{eff}})^6 = 2R_0^6$$

where R_0 is defined by

$$R_0 = [(8.75 \times 10^{-5})n^{-4}\phi_D J_{DF}\kappa^2]^{1/6} \text{ Å} \tag{3}$$

in which n is the refractive index of the intervening medium, normally given a value of 1.4 in biological systems (Beardsley & Cantor, 1970), ϕ_D is the quantum yield of IAEDANS in the absence of the acceptor, and the overlap integral (J_{DF}) is a measure of the overlap between the donor emission and the acceptor excitation spectra. The extent of the overlap is shown in Figure 4. Finally, κ^2 is the orientation factor, which has a value of 2/3 when the two dyes are freely rotating but can in principle have a value between 0 and 4 (Dale & Eisinger, 1974).

Assuming random labeling of the DNA 3′ ends, the fractions of DNA fragments containing different numbers of fluorescein molecules are shown in Table I, where f_a is the fractional fluorescein content of the nucleosomes, defined as the number of dye molecules per DNA 3′ end. The observed efficiency of energy transfer is equal to the weighted sum of the individual transfer efficiencies E_1, E_2, and E_3 of the various species of nucleosomes in this population:

$$E_0 = f_a^2E_1 + 2f_a(1 - f_a)E_2 + (1 - f_a)^2E_3 = f_a^2\frac{2R_0^6}{2R_0^6 + R^6} + 2f_a(1 - f_a)\frac{R_0^6}{R_0^6 + R^6} \tag{4}$$

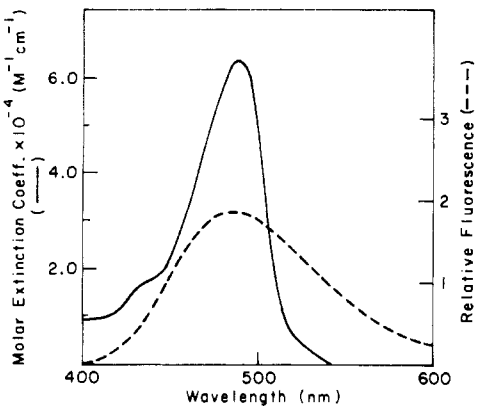

FIGURE 4: Corrected emission spectrum (- - -) of nucleosomes containing the donor IAEDANS and corrected excitation spectrum (—) of nucleosomes containing the acceptor fluorescein.

Table I: Probabilities and Properties of Species Present in Partially Labeled Compact Nucleosomes

species	probability	ϵ_{490}^{eff}	$(R_0^{eff})^6$	E^{eff}
(1) F, F	f_a^2	$2\epsilon_{490}$	$2R_0^6$	$2R_0^6/(2R_0^6 + R^6)$
(2) F	$2f_a(1-f_a)$	ϵ_{490}	R_0^6	$R_0^6/(R_0^6 + R^6)$
(3)	$(1-f_a)^2$	0	0	0

When the energy transfer efficiency $E_c = E_o/f_a$ corrected for fractional labeling in the one donor–one acceptor case is substituted for the observed efficiency E_o, eq 4 can be reduced to

$$R = R_0\left[\frac{1}{E_c} - \frac{3}{2} + \frac{[E_c^2 + (4 - 8f_a)E_c + 4]^{1/2}}{2E_c}\right]^{1/6} \quad (5)$$

Upon unfolding of the nucleosome, the donor sites move farther apart, and one must consider the possibility that each donor is within effective energy transfer range of at most one acceptor. In this case the nucleosome population can be effectively described by species 2 and 3 in Table I, with the respective probabilities f_a and $1 - f_a$, and the distance R can be determined by the simple rearrangement of the Förster equation:

$$R = R_0\left(\frac{1}{E_c} - 1\right)^{1/6} \quad (6)$$

(3) Quantitation of DNA Labeling. The molar extinction coefficient (ϵ_{490}) of the fluorescein-labeled DNA and the stoichiometry of DNA labeling were determined in the denaturing conditions of 2 M NaCl and 5 M urea. We used high salt–urea denaturing conditions for two reasons: (a) nucleosomes are completely dissociated, with little or no remaining DNA–protein interactions, and (b) the high urea concentration reduced the emission anisotropy of fluorescein from ~0.09 for F nucleosomes in physiological ionic strength to 0.03 ± 0.01, a value very similar to that obtained for the reference compound β-mercaptoethanol–fluorescein (ME–IAF).

The value of 72 200 M^{-1} cm^{-1} was obtained for the visible molar extinction coefficient (ϵ_{490}) of ME–IAF in 2 M NaCl, 5 M urea, and TE by three independent absorption measurements. Because of the above stated reasons, the molar extinction coefficients of DNA–IAF in the variety of ionic strength conditions used in this study were determined relative to the value of 72 200 M^{-1} cm^{-1} for DNA–IAF in the high salt–urea denaturing medium. As shown in Table II, the ϵ_{490} of fluorescein-labeled DNA (as well as F nucleosomes) was very dependent on the ionic strength, probably indicating a changing interaction between the negatively charged dye molecule and the bases of the double helix.

Table II: Summary of the Optical Properties of 146-H3 Nucleosomes

condition	I_s (mM)	ϵ_{490}^F (M^{-1} cm^{-1})[a]	λ_{max}^D (nm)	ϕ_D	$R_0^{2/3}$ (Å)[b]	r_F[c]	r_D[c]
1/50 × TE	0.32	39 900	490 ± 2	0.42	42.7	0.09	0.16
1/12 × TE	1.33		487 ± 2				
TE	16	66 800	476 ± 2	0.61	49.5	0.09	0.21
0.1 M NaCl and TE	116	75 900	476 ± 2	0.61	50.6		
0.35 M NaCl and TE	366	79 000	477 ± 2	0.55	50.0	0.09	0.21
0.60 M NaCl and TE	616	79 000	484 ± 2	0.20	42.3	0.09	0.15

[a] The fluorescein molar extinction coefficients (M^{-1} cm^{-1}) were determined relative to a value of 72 200 M^{-1} cm^{-1} in 5 M urea, 2 M NaCl, and TE; see Experimental Procedures for details. [b] The critical distance $R_0^{2/3}$ was calculated through eq 3 assuming a value of 2/3 for the orientation factor κ^2. [c] r_F and r_D are the emission anisotropies (±0.01) of nucleosomal fluorescein and IAEDANS, respectively.

The stoichiometry of fluorescein (f_a = IAF/DNA 3′ end) in the energy transfer samples was determined by fluorescence; the excitation wavelength was 490 nm and fluorescence was monitored at 520 nm. Since fluorescein has a pK_a = 6.46 (Mercola et al., 1972), slightly higher pH values were used in these measurements to ensure that all residues were ionized. The fractional fluorescein content of the nucleosomes was determined by (1) measurement of A_{260} in TE for the determination of nucleosome concentration, (2) 3× dilution with 7.5 M urea, 3 M NaCl, and 50 mM Tris-HCl, pH 8.9 (or pH 9.4 carbonate buffer), and (3) measurement of fluorescence relative to ME–IAF in an identical medium.

(4) Dipole–Dipole Orientation Factor. The major source of uncertainty in the calculation of R_0 is the value of κ^2, which is not accessible to direct measurement. In principle, κ^2 can range from 0 to 4. However, using emission anisotropy measurements one can determine the maximum and minimum values of κ^2 by the method developed by Dale & Eisinger (1974). We conducted fluorescence polarization measurements by exciting at 380 and 490 nm and monitoring emission at 480 and 520 nm for IAEDANS- and fluorescein-labeled nucleosomes, respectively. The method for the extraction of emission anisotropy from polarization data is described elsewhere (Fairclough & Cantor, 1978).

Analytical Procedures. Polyacrylamide gel electrophoresis of DNA and nucleosomal particles was performed as described by Maniatis et al. (1975) using 5% slab gels in Tris–borate–EDTA buffer. Histone proteins were analyzed by electrophoresis on 15% polyacrylamide–0.1% $NaDodSO_4$ gels containing a 5% stacking gel as described by Laemmli (1970). For visualization of the fluorescein and IAEDANS emission, the gels were photographed under short UV excitation with a yellow-green filter prior to staining. Digestions with DNase I (Worthington) were performed by standard procedures (Garel & Axel, 1976). The resulting DNA fragments were analyzed by electrophoresis on 10% acrylamide gels containing 7 M urea and subsequent staining with ethidium bromide.

Results

Properties of Fluorescent Nucleosomes. Nucleosomal core particles were formed by the association of 146-bp bulk DNA

Table III: Energy Transfer Results, 146–H3 vs. Ionic Strength

condition	I_s (mM)	f_a	E_o	R/R_0[a,d]	$R^{2/3}$ (Å)[a,d]
		(a) Compact Form[a]			
TE	16	0.253[b]	0.187 ± 0.015	1.071 ± 0.024	53.0 ± 1.2
		0.156	0.138 ± 0.021	1.023 ± 0.050	50.6 ± 2.5
0.1 M NaCl and TE	116	0.156	0.151 ± 0.015	0.993 ± 0.035	50.2 ± 1.8
0.35 M NaCl and TE	366	0.253	0.194 ± 0.019	1.059 ± 0.057	53.0 ± 2.8
		0.156	0.150 ± 0.018	0.995 ± 0.043	49.8 ± 2.1
80 μM $MgCl_2$ and 1/50 × TE		0.253	0.160 ± 0.008	1.119 ± 0.015	(55.4 ± 1.0)[c]
		(b) Low-Salt Form			
1/50 × TE	0.32	0.253[b]	0.070 ± 0.023	1.174 ± 0.079[d]	50.1 ± 3.4[d]
				1.348 ± 0.076[a]	57.6 ± 3.2[a]
		(c) High-Salt Form[d]			
0.60 M NaCl and TE	616	0.253	0.047 ± 0.018	1.279 ± 0.085	54.1 ± 3.6
		(d) Other			
1/12 × TE	1.3	0.253	0.131 ± 0.023	0.988 ± 0.062[d]	
				1.176 ± 0.054[a]	

[a] The distance calculations were described under Experimental Procedures. Equation 5 was used for both the compact form and the higher limit of the low-salt form. See the text for details. [b] Averages of four separate experiments. [c] Assuming an $R_0^{2/3}$ equal to the 16 mM I_s case. [d] The distance calculations were described under Experimental Procedures. The lower limit for the H3–DNA distance in the low-salt nucleosome, as well as the distance in the high-salt form, was calculated through eq 6. See the text for details.

fragments with the four core histones. Using DNA and protein preparations containing different fluorescent labels at various specific sites, we have prepared nucleosomal particles with a variety of different emission properties. One set of samples, 146–H3, were prepared by the reconstitution of 146-bp DNA labeled at both 3′ termini with 5-(iodoacetamido)fluorescein (DNA–IAF) and CE core histones modified at the cysteine-110 of histone protein H3 with the environmentally sensitive dye [^{3}H]-5-[[(iodoacetamido)ethyl]amino]naphthalene-1-sulfonic acid (H3–IAEDANS) (Figure 1A–C). Samples containing the three different combinations of these two fluorescent probes, namely, DF, D, and F, were prepared as described under Experimental Procedures.

The fidelity of the internal structure of the 146–H3 modified nucleosomes was tested by several methods. The fluorescent particles exhibited the same electrophoretic mobility as native nucleosomes on polyacrylamide gels. Their sedimentation properties as judged on sucrose gradients were identical with those of native trimmed core particles (Figure 2). The circular dichroism spectrum of the doubly labeled DF nucleosome was measured. The molar ellipticity at 282 nm was 1746 ± 96 deg cm^2/dmol of phosphate as compared to 8240 deg cm^2/dmol of phosphate for protein-free nucleosomal DNA and a reported value of 1800 deg cm^2/dmol of phosphate for native core particles (Klevan & Crothers, 1977). In addition, the DNase I digest of labeled nucleosomes showed the standard 10-bp repeat pattern in the cleavage of the nucleosomal DNA.

Similarly a second set of fluorescent-labeled nucleosomes, 146–H4, containing the donor IAEDANS at the single cysteine (residue 73) of *A. lixula* sperm histone H4 (Figure 1A,B) were prepared. The nucleosomes were reconstituted with fluorescein-labeled 146-bp DNA as previously, with a mixed pool of histones: *Arbacia* H3 and H4 and chicken erythrocyte H2A–H2B. The amount of aggregation was much greater than in reconstitutions with CE core histones, and at times the 10.3S nucleosome fractions from primary sucrose gradients had to be pooled, concentrated, and resedimented for further purification. The higher degree of aggregation and lower yields of purified 146–H4 nucleosomes were probably due to the error involved in the input molar ratio of the separate histone proteins. However, the gel electrophoretic mobility (Figure 5) and also the sedimentation properties of the purified nucleosomes were indistinguishable from those of native CE nucleosomes, and DNase I digestion experiments resulted in the characteristic banding pattern on denaturing DNA gels.

Energy Transfer Experiments with 146–H3 Nucleosomes. (*1*) *Physiological Conditions.* We carried out singlet–singlet energy transfer experiments using 146–H3 reconsituted nucleosomes which contained the donor IAEDANS on the cysteine residue of histone H3 and the acceptor fluorescein on the DNA termini. The fluorescence experiments performed at the three ionic strengths (I_s) 16, 116, and 366 mM yielded very similar emission spectra, indicating very closely related nucleosome structures under these nearly physiological salt concentrations. The emission spectra from a typical energy transfer experiment with 146–H3 nucleosomes in TE buffer (16 mM I_s) are shown in Figure 3. The obvious quenching of the H3–IAEDANS emission indicates the occurrence of nonradiative energy transfer to the fluorescein acceptor molecules. The observed efficiency of energy transfer E_o at 16 mM I_s was 0.187 ± 0.015 and 0.138 ± 0.021 for the two nucleosome preparations used. Table III(a) summarizes the data from these experiments. Each of the reported values of E_o is the average of at least two separate experiments. The difference in the E_o between the two preparations is due to differences in fluorescein stoichiometry as indicated in Table III(a).

Because of the proximity of the two H3–IAEDANS molecules in the compact conformational state of the nucleosome and the probable twofold symmetry of the core particle (Finch et al., 1977), both donor molecules must be positioned on the dyad axis, equidistant from the DNA termini. As described under Experimental Procedures, eq 5 was derived for such a case. When this equation was used to analyze the experimentally determined E_o, the resulting ratio R/R_0 at 16 mM I_s was 1.071 ± 0.024 and 1.023 ± 0.050 for the two nucleosome preparations. Notice that although the values for E_o were significantly different, the resulting values for R/R_0 are the same within experimental error.

Assuming a value of 2/3 for the orientation factor κ^2, a value of 50–53 Å was determined for the distance between the cysteine-110 of histone H3 and the DNA 3′ termini in the compact nucleosome. More thorough (Dale & Eisinger, 1974) analysis of κ^2 and the resultant limitations on the calculated distance R will be discussed in a later section.

(*2*) *Low-Salt Transition.* The nucleosome low-salt transition has recently been studied by a variety of physical methods (Gordon et al., 1978; Dieterich et al., 1979; A. E. Dieterich,

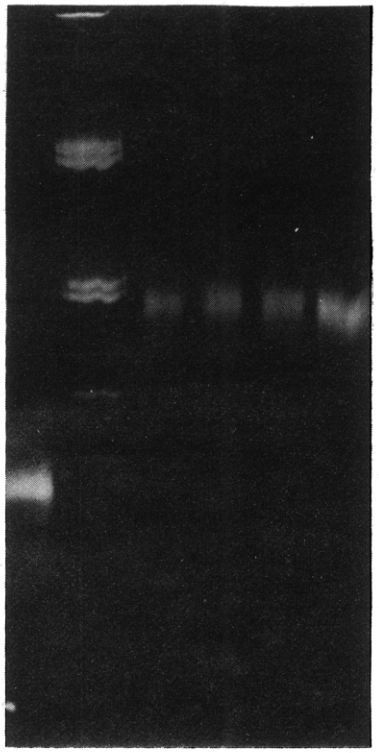

FIGURE 5: Five percent polyacrylamide gel of 146–H4 nucleosomes. From left to right: 146-bp DNA used for reconstitution; *Hae*III digest of ColE1 plasmid DNA; nucleosomal particles reconstituted with *A. lixula* sperm histones H3 and H4 and chick erythrocyte histones H2A and H2B containing IAEDANS-labeled H4 and fluorescein-labeled DNA (DF), unlabeled H4 and fluorescein-labeled DNA (F), and IAEDANS-labeled H4 and unlabeled DNA (D); native CE trimmed nucleosomes. The fragments of the *Hae*III ColE1 digest visible on this gel are 1100, 1050, 2 × 910, 2 × 440, 2 × 400, 250, 178, 85, and 2 × 70 bp. Electrophoresis was from top to bottom.

H. Eshaghpour, D. M. Crothers, and C. R. Cantor, unpublished experiments; Wu et al., 1979). It has been shown that at very low ionic strengths the 146-bp nucleosome unfolds, exposing the cysteine residue of histone H3 to the solvent (Dieterich et al., 1979), leaving the DNA cylindrically wound in 0.9 turn about an enlarged cylinder (Wu et al., 1979). We carried out energy transfer experiments at 0.32 mM ionic strength (1/50 × TE) to examine further the internal organization of the nucleosome in the low-salt conformation.

The emission spectra of a set of 146–H3 nucleosome samples dialyzed to 0.32 mM I_s exhibited drastically reduced fluorescence of both IAEDANS (Table II) and fluorescein. The calculated E_o (0.07 ± 0.023) was much less than that determined for the compact state of the nucleosome [Table III(b)].

In the absence of detailed information concerning the internal structure of nucleosomes in low salt, we can only determine a range for the distance between the H3 cysteines and the DNA ends. At one extreme is the instance where each IAEDANS molecule can transfer energy to only a single fluorescein. The other limit is attained if each donor molecule is equidistant to the two ends of the DNA, a situation similar to that in the compact nucleosome. Therefore, the limiting distances can be calculated by the two methods of analysis described under Experimental Procedures. Using the basic Förster equation, eq 6, we calculated the H3–DNA distance ($R^{2/3}$) for the one donor–one acceptor case to be 50.1 ± 3.4 Å. Alternatively, by use of eq 5, the other limiting value (one donor–two acceptor case) of the H3–DNA distance ($R^{2/3}$) was determined to be 57.6 ± 3.2 Å. We can therefore place the actual distance between each H3 cysteine and its corresponding DNA 3′-hydroxyl group to be within the range of 50–58 Å in the low-salt unfolded form of the nucleosome, a distance not very different from that measured for the compact nucleosome. Therefore, the large difference between the values of E_o for the compact and low-salt form of the nucleosome is primarily due to the drastic change in the optical properties of the donor–acceptor pair (Table II).

Table IV: Energy Transfer Results, 146–H3 vs. Urea[a]

urea (M)	λ_{max}^{D} (nm)	ϕ_D[b]	r_D	r_F	E_o	R/R_o
0	476	0.61	0.22	0.09	0.187 ± 0.015	1.071[c]
1.5	477	0.61	0.22	0.07	0.172 ± 0.015	1.097[c]
3.0	484	0.43	0.17	0.07	0.085 ± 0.018	1.120[d]
						1.296[c]
5.0	492	0.30	0.11	0.03	0.086 ± 0.021	1.117,[d]
						1.292[c]

[a] The fluorescein stoichiometry $f_a = 0.253$. [b] The quantum yield of IAEDANS (ϕ_D) at each state was determined relative to the value of 0.61 by using their fluorescence intensities at the λ_{max}. [c] Calculated by eq 5. [d] Calculated by eq 6.

(*3*) *Refolding of Nucleosomes.* The emission spectrum and the resulting E_o value changed back to those characteristic of the compact nucleosome upon increasing the ionic strength in a solution of unfolded nucleosomes to greater than 4–5 mM by direct addition of 100 × TE buffer. If, however, the ionic strength was increased to 1–2 mM, the midpoint of the low-salt transition, the system behaved in a complex fashion. The H3–IAEDANS emission spectrum was very similar to the low-salt form with a λ_{max} of ~490 nm, while the E_o [Table III(d)] was nearly the same as that found for the compact nucleosome [Table III(a)]. This observation suggests the existence of intermediate states and provides evidence against a simple one-step kinetic pathway for the folding of the nucleosome. Further evidence for intermediate states has been found by analysis of the reaction kinetics (A. Deiterich and C. Cantor, unpublished experiments).

It was also found that the low-salt unfolded nucleosome could be converted to its compact form by very small amounts of added Mg^{2+}. Addition of 80 μM $MgCl_2$ to nucleosomes in 1/50 × TE (200 μM Tris-HCl and 40 μM EDTA) resulted in emission properties characteristic of the compact nucleosome [Table III(a)].

(*4*) *Unfolding in 0.6 M NaCl.* Measurement of the emission spectra of the appropriate 146–H3 samples in 0.6 M NaCl yielded the value of 0.047 ● 0.018 for the observed efficiency of energy transfer [Table III(c)]. Due to the large H3–H3 distance in 0.6 M salt (>70 Å; Dieterich et al., 1979), the system was represented by a one donor–one acceptor model and eq 6 was used in the determination of the H3–DNA distance. Because of the highly quenched H3–IAEDANS emission under these conditions (Q = 0.2; Table II), the calculated distance $R^{2/3}$ was 54.1 ± 3.6 Å, still very similar to the previously determined distances.

The unfolding caused by 0.6 M NaCl is very slow, and an overnight incubation period is necessary for equilibrium to be attained. Fluorescence measurements up to several hours after the addition of NaCl yielded an H3–IAEDANS spectrum and E_o values similar to those obtained for the compact nucleosome.

(*5*) *Unfolding in Urea.* The distance between the DNA termini and the H3 cysteines was also monitored as a function of urea concentration. A set of samples in TE buffer showing characteristic emission properties was adjusted initially to 1.5, then to 3.0, and finally to 5.0 M urea by the addition of a stock 10 M urea and TE solution. The emission spectrum in each

Table V: Energy Transfer Results, 146-H4 Nucleosomes[a]

form	condition	E_o	R/R_o	$R_o^{2/3}$ (Å)	$R^{2/3}$ (Å)
low-salt unfolded	1/50 × TE (0.32 mM I_s)	0.251 ± 0.028	≤0.72	43	≤31
compact	1 × TE (16 mM I_s)	0.243 ± 0.011	≤0.67	47	≤32

[a] $\lambda_{max}^{D} \simeq 484$ nm, $\phi_D = 0.55 \pm 0.05$, $f_a = 0.253$, and appropriate values of ϵ_{490}^{F} were used from Table II. The distance (R) was calculated by assuming a value of 2/3 for κ^2 and using the basic Förster equation, eq 6.

instance was obtained after a minimum incubation period of 6 h. Table IV summarizes the results obtained from these experiments. At 1.5 M urea the spectra and energy transfer efficiency corresponded to a compact nucleosomal conformation. Many changes were observed, however, upon increase of the urea concentration to 3 M and beyond. The H3–IAEDANS emission spectrum was quenched drastically, the λ_{max} was shifted to 484 nm, and the value of E_o dropped to 0.085 ± 0.02. The emission anisotropy of H3–IAEDANS (r_D) dropped from 0.22 in the native nucleosome to 0.11 in 5 M urea. All these changes indicate an extensive unfolding of the nucleosome in 3–5 M urea. Assuming a critical distance ($R_0^{2/3}$) similar to that in 0.6 M NaCl, the H3–DNA distance in the urea-denatured nucleosome is ~47–55 Å, which is still very close to the H3–DNA distance measured in other conditions.

The reversibility of the urea denaturation was checked by an overnight dialysis of the 5 M urea samples against two changes of TE buffer. All resulting emission properties of the samples, including the value of E_o and IAEDANS emission anisotropy (r_D), returned to those of the compact conformational state of the nucleosome (data not shown).

Energy Transfer Experiments with 146–H4 Nucleosomes. The emission properties of nucleosomes containing H4–IAEDANS were relatively insensitive to nucleosome unfolding. The distance between the DNA termini and residue 73 of sperm histone H4 from *A. lixula* was probed by singlet–singlet energy transfer. The observed efficiency of energy transfer E_o for these nucleosomes was very high in both 0.32 and 16 mM ionic strength, indicating a small distance (≤32 Å) between the probes. A summary of the results of these experiments is provided in Table V.

Orientation Factor κ^2. The range of values for the geometrical factor κ^2 can be limited by the use of fluorescence anisotropy measurements according to Dale & Eisinger (1974). Using the compact form of the nucleosome, which showed the highest amount of polarization for both labels, we determined the extreme estimates of κ^2. These are summarized in Table VI.

The emission anisotropy (r) is related to the limiting anisotropy (r_0) by

$$r = \frac{r_0 \tau_R}{\tau_R + \tau} \qquad (7)$$

where τ is the fluorescence lifetime and τ_R is the rotational correlation time of the particle. Fluorescein and IAEDANS have fluorescence lifetimes (τ) of 5 and 22 ns, respectively, in the compact nucleosome. Since the nucleosome has a rotational correlation time (τ_R) of 145 ns, the limiting anisotropy r_0 will be very close to the measured anisotropy (Dieterich et al., 1979). As shown in Table VI, the half-angle of the conical volume, within which the chromophore has rotational freedom, was determined (Dale & Eisinger, 1974; Fairclough & Cantor, 1978). Values of 33 and 54° were obtained for IAEDANS and fluorescein, respectively. Thereafter, the range of κ^2 values was computed through the graphical analysis of Dale & Eisinger (1974). In the compact nucleosome, the geometrical factor κ^2 is restricted to values between 0.3 and 0.69, resulting in a range of 43.3–49.8 Å for the critical distance R_0. The actual H3 to DNA distance, therefore, lies between the extreme limits 45 and 52 Å, compared to the value ($R^{2/3}$) of 50–53 Å. The above range of values was determined disregarding the parallel–parallel orientation of the transition dipoles, because of their low statistical probability (Hillel & Wu, 1976). The values shown in parentheses in Table VI represent the limits for this orientation.

Table VI: Analysis of the Orientation Factor κ^2 for the Compact 146–H3 Nucleosome

parameter	symbol	donor (IAEDANS)	acceptor (fluorescein)
emission anisotropy	r	0.21 ± 0.01	0.09 ± 0.01
limiting anisotropy	r_0	0.24	0.09
dynamic depolarization factor[a]	$\langle d' \rangle$	0.60	0.23
cone half-angle for volume reorientation (deg)	Ψ	33	54
range of $\langle \kappa^2 \rangle$		$0.3 \leq \kappa^2 \leq 0.69$	(2.2)[b]
range of R_0 (Å)		$43.3 \leq R_0 \leq 49.8$	(60.4)[b]
range of R (Å)		$45.3 \leq R \leq 52.1$	(63.2)[b]

[a] The dynamic depolarization factor is defined by $\langle d' \rangle = r_0/0.4$. [b] Values in parentheses correspond to models with a direct in-line orientation of transition dipoles. The arrangement has a very low statistical probability and we exclude it from consideration [see Hillel & Wu (1976)].

Despite the large changes observed in the polarization of the two dyes in the different forms of the nucleosome (Tables II and IV), the calculated distances ($R^{2/3}$) remained constant. The $R^{2/3}$ values reported throughout this study, therefore, provide good estimates of the actual distances.

Discussion

The Cysteine Residue of Histone H3 Lies Near the Center of the Nucleosome. Our results show that the DNA 3′ termini are located 50–53 Å from cysteine-110 in chick erythrocyte histone H3 reconstituted into nucleosomal core particles with 146 base pair DNA. Considering both the 110-Å diameter of cylindrical models of the core particle (Finch et al., 1977; Pardon et al., 1977; Crothers et al., 1978) and the restriction that the two cysteine residues lie on the dyad axis because of their proximity (Camerini-Otero & Felsenfeld, 1977; Zama et al., 1978; Dieterich et al., 1979), we conclude that the two cysteine groups must be located very near the center of the core particle.

The H3 to DNA Distance Remains Constant Regardless of the Conformation of the Nucleosome. We have probed the internal organization of the nucleosome by determining the H3 to DNA distance in several nonphysiological conditions, including very low and high salt and urea. Although the value of the observed efficiency of energy transfer (E_o) was much lower in 0.32 mM ionic strength, the resulting H3–DNA distance was in the range of 50–58 Å, nearly unchanged from the compact form of the nucleosome. Furthermore, approximately the same H3–DNA distance was measured for nucleosomes in both 0.6 M NaCl and 5 M urea.

In agreement with other recent physicochemical studies, our fluorescence measurements suggest gross structural changes in the nucleosome under the tested conditions. It is therefore striking that the distance between the two specific loci, the 3′ DNA end and H3 cysteine, remained essentially unchanged

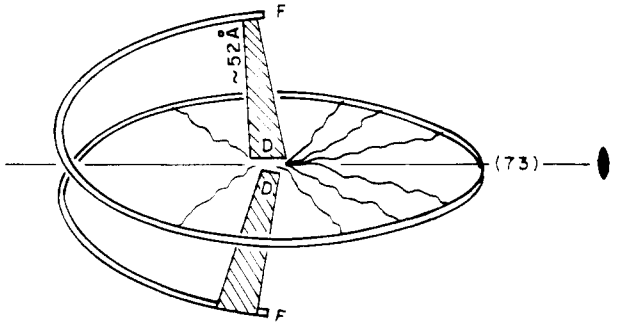

FIGURE 6: Schematic drawing of the compact nucleosome. The crosshatched areas represent the very strong and stable binding of the DNA termini and segments of the histone proteins containing cysteine-110 of CE histone H3 (D) and cysteine-73 of *A. lixula* sperm histone H4. The wavy lines represent more flexible histone–DNA interactions. The DNA termini and the central part of the DNA (around residue 73) have been shown by cross-linking experiments (Mirzabekov et al., 1978) to be in proximity with histones H3 and H4.

under all denaturing conditions. Since it is known that at least parts of the two H3 proteins within each nucleosome move apart in low and high ionic strengths (Dieterich et al., 1979), we conclude that the C-terminal region of each histone H3 must be tightly bound to one DNA end, with the protein and DNA moving in unison during the unfolding process. The finding of H3–DNA cross-links at the DNA termini by Mirzabekov et al. (1978) provides chemical support for our observations.

A Very Strong Contact Is Present between Each DNA End and a Complex of the C-Terminal Regions of Histones H3 and H4. In a parallel series of experiments, the distance between the DNA termini and the single cysteine of *A. lixula* sperm histone H4 was measured. The energy transfer data showed that the two labeled sites are closer than 32 Å in both the compact and the low-salt unfolded nucleosomes. This observation is in fact not surprising, considering the constancy of the H3–DNA distance and the seemingly strong interaction between the histone proteins H3 and H4 (Kornberg & Thomas, 1974; Roark et al., 1974; D'Anna & Isenberg, 1974; Rubin & Moudrianakis, 1975). Furthermore, Böhm et al. (1977) have studied the interaction between several partial peptides from histones H3 and H4 by nuclear magnetic resonance spectroscopy and have concluded that the regions important for H3–H4 complex formation are defined by residues 42–120 in histone H3 and residues 38–102 in histone H4. Note that these two interacting peptides contain both of the modified residues in our experiments.

Unfolding of Nucleosomes by Alteration of the Interactions Involving Histone N-Terminal Regions. It is obvious that some of the protein–protein and protein–DNA interactions must be altered as the nucleosome unfolds. Maintenance of the very stable contact(s) between the DNA termini and the C-terminal regions of the histones H3 and H4 may constitute the intermediate necessary for the refolding of the nucleoprotein complex from its unfolded conformational states.

It has been shown that trypsinized histones, lacking their N-terminal regions, are capable of folding DNA into a nucleosome-type particle (Whitlock & Stein, 1978). Necessarily, other histone–DNA contacts, most notably those of H3 and H4, must be instrumental in the organization of nucleosomes. In addition, since trypsinized nucleosomes exhibit many of the characteristics of native nucleosomes (Lilley & Tatchell, 1977; Whitlock & Simpson, 1977) and because histone proteins are modified in vivo primarily at their N-terminal regions (Allfrey, 1977), it is likely that alterations in the interaction between the N termini of the histones and the central part of the DNA (Whitlock & Simpson, 1977) are partially responsible for the changes in the conformation of nucleosomes.

Using the general model proposed for the structure of the DNA in the compact nucleosome (Finch et al., 1977; Pardon et al., 1977; Crothers et al., 1978), we have prepared a schematic drawing (Figure 6) to show the two types of interaction present between the DNA and histone proteins H3 and H4 within the nucleosome. The solid hatched regions represent the very strong and stable interaction between each DNA end (F) and segments of the protein core containing the cysteine residues on chick erythrocyte histone H3 (D) and sea urchin histone H4. During the unfolding of the nucleosome, the interactions represented by the solid hatched regions are maintained, while the other contacts, as shown by the wavy lines, are at least partially altered.

Origin of Trimmed Core Particles. If parts of the H3–H4 complex are in very close association with segments of the DNA ~140 bp apart, they could render unusual protection to the nucleosomal DNA in chromatin, giving rise to the in vitro produced 146-bp core particles. In support of this interpretation, we have detected unusual resistance to exonuclease III at positions corresponding to these binding sites in an artificial nucleosome formed with a 178-bp DNA restriction fragment (H. Eshaghpour and D. M. Crothers, unpublished experiments).

Acknowledgments

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Cyclo(L-prolylglycyl)$_3$ and Its Sodium, Potassium, and Calcium Ion Complexes: A Raman Spectroscopic Study†

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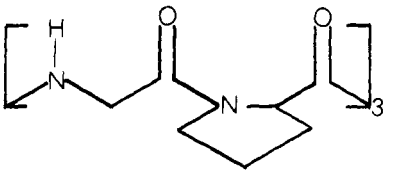
ABSTRACT: Raman spectra of the cyclic hexapeptide cyclo(L-prolylglycyl)$_3$ and its Na^+, K^+, and Ca^{2+} complexes are reported for the solid state and for samples in solution. Model compounds and N-deuteration were used to aid mode identification. Spectra of the uncomplexed ionophore in solution are consistent with previously proposed solution conformations and permit the identification of spectral lines characteristic of proline-containing peptide bonds in the trans and the cis conformations. Upon cation complexation the prolyl carbonyl stretch bands sharpen and upshift 20–30 cm^{-1} (to 1690–1700 cm^{-1}). The glycyl carbonyl stretch band is unaffected by Na^+ complexation, upshifted ~15 cm^{-1} by K^+ complexation, and downshifted ~20 cm^{-1} (to 1619 cm^{-1}) by Ca^{2+} complexation. Arguments supporting the involvement of prolyl carbonyl groups in cation complexation are noted. Spectra of the Na^+ complex of the tetramer cyclo(L-prolylglycyl)$_4$ suggest an asymmetric structure.

Due to their biological activity and structural simplicity, considerable interest has been aroused in the conformations and ion-binding characteristics of synthetic cyclic polypeptides. In particular, the cyclic hexapeptide cyclo(L-prolylglycyl)$_3$ (see Chart I), hereinafter abbreviated c(PG)$_3$, is known (Madison et al., 1974; Deber et al., 1976) to bind Li^+ and Na^+ selectively over K^+ and Rb^+. It also forms several complexes of different stoichiometry with Mg^{2+}, which it binds selectively over Ba^{2+} and Ca^{2+}. These complexes have been studied by proton and ^{13}C nuclear magnetic resonance (NMR) (Bartman et al., 1977), circular dichroism, and conformational minimum en-

Chart I



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